U.PORTO

DOUTORAMENTO

MEDICINA

Doenças do Interstício Pulmonar: do fenótipo ao genótipo

António Manuel Martins de Morais

D

2015

Dissertação de candidatura ao grau de Doutor
apresentada à Faculdade de Medicina da Universidade do Porto

Orientadores:

Professor Doutor José Agostinho Marques
Departamento de Medicina
Faculdade de Medicina da Universidade do Porto, PT

Professor Doutor Luís Delgado
Serviço e Laboratório de Imunologia
Faculdade de Medicina da Universidade do Porto, PT

Artigo 48º, Parágrafo 3º

"A Faculdade não responde pelas doutrinas expendidas na dissertação."

Regulamento da Faculdade de Medicina da Universidade do Porto
Decreto-Lei nº 19337 de 29 de Janeiro de 1931

JÚRI DAS PROVAS DE DOUTORAMENTO

Presidente Diretora da Faculdade de Medicina (por delegação reitoral)

Vogais

Doutor Hilário Lourenço Nunes
Professor Catedrático da Université Paris 13, France

Doutor Carlos Manuel da Silva Robalo Cordeiro
Professor Associado da Faculdade de Medicina da Universidade de Coimbra

Doutora Deolinda Maria Valente Alves Lima Teixeira
Professora Catedrática da Faculdade de Medicina da Universidade do Porto

Doutor Francisco Fernando da Rocha Gonçalves
Professor Catedrático da Faculdade de Medicina da Universidade do Porto

Doutor José Agostinho Marques Lopes
Professor Catedrático da Faculdade de Medicina da Universidade do Porto

Doutor João Carlos Winck Fernandes Cruz
Professor Afiliado da Faculdade de Medicina da Universidade do Porto

CORPO CATEDRÁTICO
DA FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

Professores Efectivos

Manuel Alberto Coimbra Sobrinho Simões
Maria Amélia Duarte Ferreira
José Agostinho Marques Lopes
Patrício Manuel Vieira Araújo Soares Silva
Daniel Filipe Lima Moura
Alberto Manuel Barros da Silva
José Manuel Lopes Teixeira Amarante
José Henrique Dias Pinto de Barros
Maria Fátima Machado Henriques Carneiro
Isabel Maria Amorim Pereira Ramos
Deolinda Maria Valente Alves Lima Teixeira
Maria Dulce Cordeiro Madeira
Altamiro Manuel Rodrigues Costa Pereira
Rui Manuel Almeida Mota Cardoso
António Carlos Freitas Ribeiro Saraiva
José Carlos Neves da Cunha Areias
Manuel Jesus Falcão Pestana Vasconcelos
João Francisco M. A. Lima Bernardes
Maria Leonor Martins Soares David
Rui Manuel Marques Nunes
José Eduardo Torres Eckenroth Guimarães
Francisco Fernando Rocha Gonçalves
José Manuel Pereira Dias de Castro Lopes
António Albino Coelho M. Abrantes Teixeira
Joaquim Adelino C. Ferreira Leite Moreira
Raquel Ângela Silva Soares Lino

Professores Jubilados/Aposentados

Abel Vitorino Trigo Cabral
Alexandre Alberto Guerra Sousa Pinto
Álvaro Jerónimo Leal Machado de Aguiar

Amândio Gomes Sampaio Tavares
António Augusto Lopes Vaz
António Carvalho Almeida Coimbra
António Fernandes Oliveira B. Ribeiro Braga
António Germano Silva Pina Leal
António José Pacheco Palha
António Manuel Sampaio de Araújo Teixeira
Belmiro dos Santos Patrício
Cândido Alves Hipólito Reis
Carlos Rodrigo Magalhães Ramalhão
Cassiano Pena de Abreu e Lima
Daniel Santos Pinto Serrão
Eduardo J. Cunha Rodrigues Pereira
Fernando Tavarela Veloso
Francisco de Sousa Lé
Henrique José F. G. Lecour de Menezes
Jorge Manuel Mergulhão Castro Tavares
José Carvalho de Oliveira
José Fernando Barros Castro Correia
José Luís Medina Vieira
José Manuel Costa Mesquita Guimarães
Levi Eugénio Ribeiro Guerra
Luís Alberto Martins Gomes de Almeida
Manuel António Caldeira Pais Clemente
Manuel Augusto Cardoso de Oliveira
Manuel Machado Rodrigues Gomes
Manuel Maria Paulo Barbosa
Maria da Conceição F. Marques Magalhães
Maria Isabel Amorim de Azevedo
Mário José Cerqueira Gomes Braga
Serafim Correia Pinto Guimarães
Valdemar Miguel Botelho dos Santos
Walter Friedrich Alfred Osswald

Lista de Publicações

Esta dissertação é baseada nas seguintes publicações, que serão referidas no texto de acordo com os seus números romanos:

I. BTNL2 gene polymorphism associations with susceptibility and phenotype expression in sarcoidosis. António Morais, Bruno Lima, Maria José Peixoto, Helena Alves, Agostinho Marques, Luís Delgado.
Respiratory Medicine (2012) 106, 1771-1777

II. Annexin A11 gene polymorphism (R230C variant) and sarcoidosis in a Portuguese population. António Morais, Bruno Lima, Maria Peixoto, Natália Melo, Helena Alves, J. Agostinho Marques, Luís Delgado.
Tissue Antigens, 2013, 82, 186–191

III. Associations between sarcoidosis clinical course and ANXA11 rs1049550 C/T, BTNL2 rs2076530 G/A, and HLA class I and II alleles. António Morais, Bruno Lima, Helena Alves, Natália Melo, Patrícia Mota, Agostinho Marques, Luís Delgado.
BMC Pulmonary Medicine, submetido, em revisão, 2015

IV. Diagnostic value of CD103 expression in bronchoalveolar lymphocytes in sarcoidosis. Patrícia Caetano Mota, António Morais, Carmo Palmares, Marília Beltrão, Natália Melo, Ana Cristina Santos, Luís Delgado.
Respiratory Medicine (2012) 106, 1014–1020

V. Integrin aEb7 (CD103) expression in bronchoalveolar lymphocytes of patients with hypersensitivity pneumonitis. Mariana Couto, Carmo Palmares, Marília Beltrão, Sofia Neves, Patrícia Mota António Morais, Luís Delgado.
International Archives Occupational Environment Health (2015) 88:167–173

VI. Serum metalloproteinases 1 and 7 in the diagnosis of idiopathic pulmonary fibrosis and other interstitial pneumonias. António Morais, Marília Beltrão, Oksana Sokhatska, Diogo Costa, Natália Melo, Patrícia Mota, Agostinho Marques, Luís Delgado.
Respiratory Medicine (2015) doi: 10.1016/j.rmed.2015.06.003. [Epub ahead of print]

As publicações originais são disponibilizadas após permissão das respetivas revistas, detentoras dos direitos de publicação.

Resumo

Introdução

A influência de vários genes parece ser determinante para a suscetibilidade, apresentação clínica e diferentes formas de evolução nas doenças do interstício pulmonar. Por outro lado, alguns polimorfismos genéticos já descritos estão associados a vias imunopatológicas relevantes em determinadas patologias, podendo esta associação particular ser investigada como um potencial biomarcador diagnóstico.

Objetivo

Avaliar a associação dos polimorfismos genéticos descritos como mais relevantes - *ANXA11 rs1049550* C/T SNP, *BTNL2 rs2076530* G/A SNP - e alelos da classe I/II do HLA com a sarcoidose, numa população portuguesa, analisando eventuais interações gene-gene e a sua influência na evolução clínica. Foi nosso objetivo igualmente comparar a expressão de CD103 nos linfócitos T do lavado bronco-alveolar (BAL) de doentes com sarcoidose em comparação com outras doenças do interstício pulmonar (ILD), nomeadamente pneumonite de hipersensibilidade e avaliar a sua relevância como marcador diagnóstico. Avaliamos igualmente os níveis séricos das metaloproteinases (MMP) 1 e 7 no diagnóstico diferencial da Fibrose Pulmonar Idiopática (IPF) com outras ILD.

Material e Métodos

151 doentes com sarcoidose da região Norte de Portugal foram genotipados para o *BTNL2 rs2076530* G/A SNP e alelos da classe I / II do HLA e 208 para o SNP da anexina A11 *(ANXA11) rs1049550* C/T. Um grupo controlo de 150 indivíduos saudáveis foram também genotipados para o *BTNL2 rs2076530* G/A e para os alelos HLA de classe I/II e um grupo controlo de 197 doentes com as mesmas características foi genotipado para o *ANXA11 rs1049550* C/T. As amostras foram genotipadas para os SNP *ANXA11 rs1049550* C/T e *BTNL2 rs2076530* G/A utilizando ensaios de TaqMan SNPs em PCR em tempo real e de HLA de classe I / II alelos usando *primers* específicos de sequência PCR. As frequências alélicas foram comparadas com o teste do X2 (ou teste exacto de *Fisher*, quando apropriado) na análise univariada e com recurso a regressão logística, na análise multivariada. Um total de 86 doentes com ILD submetidos a BAL como componente da abordagem diagnóstica, foram divididos em 3 grupos: sarcoidose (n-41), HP (n-22) e outras ILD (n-23). Os níveis séricos de MMP 1/7 foram medidos usando a tecnologia Luminex xMAP em 139 doentes-47 IPF, 36 não-IPF Pneumonia Intersticial Usual (UIP), 14 Pneumonia Intersticial Não Específica Idiopática (iNSIP), 29 NSIP secundária (secNSIP), 13 com sarcoidose em estadio IV e 20 controlos saudáveis, cuja comparação foi efetuada usando o teste *Mann-Whitney U*. A área sob a curva (AUC/ROC) foi usada para descrever o desempenho de CD103 para o diagnóstico de sarcoidose e da MMP 1/7 para a IPF.

Resultados

Os nossos resultados confirmam a associação do alelo A do *BTNL2 rs206530* com a suscetibilidade da sarcoidose na nossa população de caucasianos. Encontramos ainda fatores genéticos de risco independentes associados a diferentes formas clínicas da doença: o alelo A do *BTNL2 rs2076530* com a doença pulmunar isolada ou sem síndrome de *Lofgren*, e o alelo ,HLA-*DRB1*03* com o síndrome de *Löfgren* ou com a resolução da doença. Verificámos também que o alelo T do *ANXA 11 rs1049550* tem um efeito protetor estatisticamente significativo quando comparados os doentes com sarcoidose com os controlos (33,2 *vs* 44,9%, p<0,001), independentemente do subgrupo de doentes com síndrome de *Löfgren*. Quanto à evolução, considerando-se 2 anos de doença, apenas o alelo HLA *DRB1*03* apresenta uma associação estatisticamente significativa com a resolução da doença (21,2% *vs* 4,9% para a doença crónica; RR=0,35; P<0,01 após a correcção de *Bonferroni* para múltiplas comparações). Não foram encontradas interações estatisticamente significativas entre os genes estudados e a evolução da sarcoidose, em qualquer das análises de regressão logística. Os doentes com sarcoidose apresentaram uma expressão CD103 significativamente reduzida nos linfócitos T do BAL, particularmente na subpopulação $CD4^+$. A razão $CD4^+CD103/CD4^+$ no BAL revelou para um ponto de corte de 0,45 o melhor desempenho diagnóstico para a sarcoidose (AUC: 0,86; sensibilidade: 81%; especificidade: 78%), incluíndo para aqueles com uma relação $CD4^+/CD8^+$ <3,5 (AUC: 0,79; sensibilidade: 75%; especificidade: 78%). Em comparação com a sarcoidose, os doentes com pneumonite de hipersensibilidade tinham um número significativamente maior de linfócitos $CD103^+$ no BAL, quer na subpopulação $CD4^+$ quer na $CD8^+$. A MMP-1 foi significativamente mais elevada na IPF que nos doentes com UIP noutro contexto que não a IPF (p=0,042) e sarcoidose (p=0,027). A MMP-7 foi significativamente maior na IPF do que nos controlos (P<0,001), não-IPF UIP (P =0,003), secNSIP (P<0,001), e sarcoidose (P<0,001). A AUC para IPF *vs* outras DPI foi de 0,63 (IC de 95%, 0,53-0,73) para MMP-1, 0,73 (IC de 95%, 0,65-0,81) para a MMP-7 e 0,74 (IC de 95%, 0,66-0,82) para MMP-1/MMP-7 combinadas. A sensibilidade e especificidade para a MMP-7 num ponto de corte=3,91 ng/mL foi de 72,3% e 66,3%, respetivamente, com um valor preditivo negativo de 82,4%.

Conclusões

Os dados obtidos confirmam a associação do alelo *BTNL2 rs2076530* com a suscetibilidade à sarcoidose nesta população portuguesa. Encontrámos fatores de risco genéticos independentes em fenótipos clinicamente distintos: *BTNL2 rs2076530* em sarcoidose com doença torácica isolada, e HLA-*DRB1*03* na síndrome de *Löfgren*. Confirmamos igualmente que o alelo *ANXA11 rs1049550** T exerce um efeito protetor estatisticamente significativo para a suscetibilidade da doença. Quanto à evolução, apenas o alelo *DRB1*03* está associado com a resolução, após 2 anos de seguimento, sem interações estatisticamente significativas com os outros polimorfismos estudados. A diminuição das células $CD4^+CD103^+$ no LBA da sarcoidose é consistente com uma redistribuição do sangue periférico e compartimentalização no pulmão e mostrou potencial como marcador de diagnóstico, nomeadamente, em casos com uma relação $CD4^+/CD8^+$<3,5. Por outro lado, os níveis de MMP-7 e MMP-1 no soro foram significativamente maiores na IPF em relação com a UIP (noutro contexto que não a IPF), a principal entidade no diagnóstico diferencial, sugerindo uma potencial utilização como um biomarcador sérico fiável neste contexto.

Abstract

Introduction

A genetic background may be involved in the susceptibility, clinical presentation and different clinical courses in interstitial lung diseases (ILD), such as sarcoidosis. The protein expressions of some genetic polymorphisms are associated with relevant pathologic pathways that sometimes, due to its association with a particular disorder, can be used as a diagnostic biomarker.

Aim

To evaluate the relationship between sarcoidosis and the most relevant associated polymorphisms described - *ANXA11 rs1049550* C/T SNP, the *BTNL2 rs2076530* G/A SNP, and HLA class I/II alleles - in a portuguese population, exploring gene-gene interactions regarding sarcoidosis outcomes. We also investigated the expression of CD103 in bronchoalveolar lavage fluid (BALF) T-lymphocytes in sarcoidosis comparatively with other ILD, namely hypersensitivity pneumonitis, and evaluated its relevance as a BALF diagnostic marker. We also intended to explore the value of serum metalloproteinases (MMP) 1 and 7 levels in the differential diagnosis of ILD.

Material and Methods

151 Caucasian patients from North region of Portugal were genotyped for *BTNL2 rs2076530 G/A* SNPs and HLA class I/II alleles and 208 for the annexin A11 *(ANXA11) rs1049550* C/T (R230C) SNP. A control group of 150 healthy subjects were also genotyped for *BTNL2 rs2076530* G/A SNPs and HLA class I/II alleles and other 197 for *ANXA11 rs1049550 C/T* SNP. Samples were genotyped for *ANXA11 rs1049550* C/T and *BTNL2 rs2076530* G/A SNPs using TaqMan Real-Time PCR Assays and for HLA class I/II alleles using PCR sequence specific primers. Allele frequencies were compared with Chi-square test in a univariate analysis (or the Fisher exact test when appropriate) and with logistic regression in a multivariate analysis. A total of 86 patients with ILD who underwent BALF as part of their initial diagnostic work-up, were enrolled into 3 groups: sarcoidosis (n-41), HP (n-22) and other ILD (n-23). Area under the receiver operating characteristic (ROC) curve (AUC) was used to describe the performance of CD103 for sarcoidosis diagnosis. MMP-1/7 serum levels were measured using Luminex xMAP technology in 139 patients- 47 IPF, 36 non-IPF Usual Interstitial Pneumonia (UIP), 14 idiopathic Nonspecific Interstitial Pneumonia (iNSIP), 29 secondary NSIP (secNSIP), 13 stage IV sarcoidosis- and 20 healthy controls, compared using the Mann-Whitney U test.

Results

BTNL2 rs206530 A allele frequencies (*Respiratory Medicine* 2012, 106: 1771-7) were significantly higher in sarcoidosis with no linkage disequilibrium with HLA-DRB1 alleles, except in the subgroup of patients with Löfgren syndrome where the determinant allele was HLA-*DRB1*03*. The A allele was also increased in those with

isolated thoracic disease, with no differences regarding radiological stages or disease evolution. HLA-*DRB1*03*, besides the association with Löfgren syndrome was significantly related with disease resolution. The frequency of the annexin *A11 rs1049550**T allele (*Tissue Antigens*, 2013, 82: 186–91) was significantly lower in sarcoidosis than in controls (33.2 vs 44.9%, P <0.001), independently of the subgroup of patients with Löfgren syndrome. Odds ratio of 0.52 and 0.44 were obtained, respectively for carriers of one (CT) and two (TT) copies normalized to the CC wild-type genotype (P <0.001). Regarding evolution, considering 2 years of evolution, only the HLA *DRB1*03* allele was significantly associated with disease resolution (21.2% vs 4.9% for chronic disease; RR = 0.35; P < 0.01 after Bonferroni correction). In the logistic regression models evaluating the association between HLA alleles and chronic sarcoidosis adjusted for *rs1049550* and *rs2076530*, only *DRB1*03* was statistically significant associated with disease resolution. No significant interactions were found in any of the logistic regression analyses (*BMC Pulmonary Medicine*, 2015, submit.).

Sarcoidosis patients presented a significantly reduced CD103 expression in BALF T lymphocytes, more pronounced in the $CD4^+$ subset (*Respir Medicine* 2012, 106: 1014-20). The BALF $CD103^+CD4^+/CD4^+$ ratio for a cutoff point of 0.45 was associated with a better diagnostic performance for sarcoidosis (AUC: 0.86 [95% confidence interval (95% CI): 0.78-0.94]; sensitivity: 81%; specificity: 78%), even for those with a $CD4^+/CD8^+$ ratio <3.5 (AUC: 0.79 [95% CI: 0.64-0.93]; sensitivity: 75%; specificity: 78%). In comparison with sarcoidosis, hypersensitivity pneumonitis patients had a significantly higher number of $CD4^+CD103^+$ and CD8+$CD103^+$ lymphocytes (*Int Arch Occup Environ Health* 2015, 88:167–73).

MMP-1 was significantly higher in IPF than non-IPF UIP (P=.042) and sarcoidosis (P = .027). MMP-7 was significantly higher in IPF than controls (P < .001), non-IPF UIP (P = .003), secNSIP (P < .001), and sarcoidosis (P < .001). AUC for IPF versus other ILD was 0.63 (95%CI, 0.53-0.73) for MMP-1, 0.73 (95%CI, 0.65-0.81) for MMP-7, and 0.74 (95%CI, 0.66-0.82) for MMP-1/MMP-7 combined. Sensitivity and specificity for MMP-7 cutoff=3.91 ng/mL was 72.3% and 66.3%, respectively, Positive Predictive Values=52.3% and Negative Predictive Values=82.4% (*Respiratory Medicine* 2015, 109: 1063-1068).

Conclusions

Our findings confirm the association of *BTNL2 rs2076530* A allele with sarcoidosis susceptibility in the Caucasian population. Moreover, we found independent genetic risk factors in clinically distinct disease phenotypes: *BTNL2 rs2076530* A allele in patients with isolated pulmonary disease or without Löfgren syndrome, and HLA-*DRB1*03* in Löfgren syndrome or disease resolution. We also confirmed that the *annexin A11 rs1049550*T* allele exerts a significant protective effect on sarcoidosis susceptibility. Regarding disease evolution, only *DRB1*03* was associated with disease resolution after 2 years' follow-up, with no statistically significant interactions with the other studied genes. The significant lower expression of $CD4^+CD103$ in sarcoidosis patients is consistent with a peripheral origin of these cells, favoring the hypothesis of redistribution from the peripheral blood and compartmentalization into the lung and showed a high potential as a diagnostic marker, namely in those with a BALF $CD4^+/CD8^+$ ratio <3.5 and in comparison with hypersensitivity pneumonitis. Serum MMP-1 and specially MMP-7 serum levels were significantly higher in IPF than in non- IPF UIP, the main entity in its differential diagnosis, suggesting a potential use as reliable IPF serum biomarker.

Abreviaturas

ATS	*American Thoracic Society*
ANXA 11	Anexina 11
BAL	Lavado Broncoalveolar
BTB	Biopsia Transbrônquica
BTNL2	Gene *Butyrophilin-like 2*
DIP	Pneumonia Intersticial Descamativa
EBUS-TBNA	Biopsia Transbrônquica Ganglionar por Ecoendoscopia Endobrônquica
ERS	*European Respiratory Society*
HLA	Sistema de Antigénios Leucocitários Humanos
HP	Pneumonite de Hipersensibilidade
HRCT	Tomografia Axial Computorizada com Cortes de Alta Resolução
IFN	Interferão
IIP	Pneumonias Intersticiais Idiopáticas
IL	Interleucina
ILD	Doenças do Interstício Pulmonar
IPF	Fibrose Pulmonar Idiopática
LAM	Linfangioleiomiomatose
LD	Desiquilíbrio de Ligação
LIP	Pneumonia Intersticial Linfocítica
MHC	Complexo Major de Histocompatibilidade
NSIP	Pneumonia Intersticial Não Específica
NPV	Valor Preditivo Negativo
OP	Pneumonia Organizativa
PLCH	Hsitiocitose Pulmonar de Células de *Langerhans*
PPFE	Fibroelastose Pleuroparenquimatosa Idiopática
PPV	Valor Preditivo Positivo
RB-ILD	Bronquiolite Respiratória com Doença Intersticial Associada
SACE	Enzima de Conversão da Angiotensina Sérica
TGF	Factor de transformação do crescimento
TNF	Factor de Necrose Tumoral
UIP	Pneumonia Intersticial Usual
WASOG	*World Association of Sarcoidosis and Other Granulomatous Disorders*

Índice

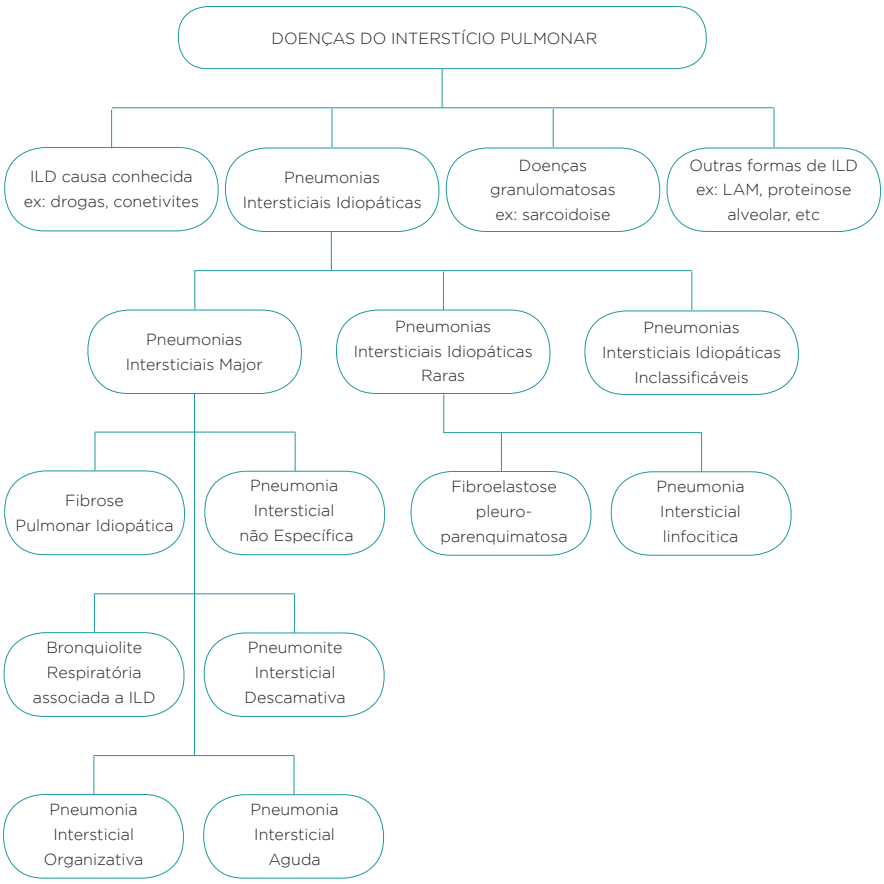
Introdução

As Doenças do Interstício Pulmonar (ILD), designadas igualmente por doenças pulmonares difusas, englobam uma série de mais de 100 doenças, que embora com uma significativa heterogeneidade na sua fisiopatologia, grau de gravidade e evolução, partilham algumas características clínicas, radiológicas e histológicas.[1-3] A sua característica principal é exatamente a distribuição pulmonar difusa, embora com padrões e distribuição distintos.[1-5] Apesar de existirem várias doenças que se podem apresentar de forma difusa, como é exemplo as de etiologia infecciosa ou neoplásica, são apenas consideradas nesta designação as doenças de carácter inflamatório e/ou fibrosante, maioritariamente de etiologia desconhecida, não sendo consideradas aquelas com aquele tipo de etiologia, ou ainda as de componente predominantemente vascular, como o caso da hipertensão pulmonar.[1-4, 6] Por outro lado, a designação intersticial é na maioria das vezes limitada, dado estas doenças envolverem igualmente as estruturas adjacentes como os bronquíolos ou os alvéolos, por vezes inclusive de forma mais significativa, o que leva alguns autores a acharem mais correta a designação destas doenças como difusas em comparação com o termo intersticial, mais tradicional. Existem dois componentes intersticiais principais, a nível das paredes e septos alveolares e o localizado na região peribroncovascular, septos interlobulares e pleura visceral.[1-3]

Embora as doenças englobadas no grupo das ILD sejam habitualmente consideradas raras, o seu conjunto contribui para uma morbilidade e mortalidade significativas, calculando-se nos Estados Unidos da América que sejam a causa de cerca de 100000 internamentos/ano e de cerca de 15% dos doentes avaliados em consultas de patologia respiratória. No entanto, estes números estarão provavelmente subavaliados, dado os registos relacionados com as ILD não serem habitualmente rigorosos, resultante destas doenças estarem ainda num período de reconhecimento, embora crescente. Por outro lado, apesar do número significativo de doenças integradas nesta designação de ILD, a maioria dos doentes manifesta apenas algumas delas, sendo as restantes extremamente raras.[1] Efetivamente, a sarcoidose, a Pneumonite de Hipersensibilidade (HP) e as Pneumonias Intersticiais Idiopáticas (IIP), nas quais se engloba a Fibrose Pulmonar Idiopática (IPF), são largamente as doenças mais frequentes, sendo por esse motivo aquelas que foram objecto primordial do nosso estudo, justificando igualmente uma maior discussão neste texto introdutório.

CLASSIFICAÇÃO DAS ILD

O elevado número de doenças associado à sua heterogeneidade, torna difícil o estabelecimento de uma classificação rigorosa, esclarecedora e consensual, existindo no entanto uma classificação proposta em 2002 pela *European Respiratory Society* (ERS) conjuntamente com a *American Thoracic Society* (ATS), que é a mais considerada e aceite (Quadro 1).[6] Segundo esta classificação, as ILD são divididas em quatro grupos principais.



Quadro 1 Proposta de classificação das ILD pela ERS/ATS publicada no consenso de 2002 e que se mantém em vigor, conjuntamente com a classificação das pneumonias intersticiais idiopáticas proposta em 2013.

Um primeiro grupo em que são incluídas as doenças com envolvimento pulmonar no contexto das doenças do tecido conjuntivo, habitualmente sob a forma de pneumonia intersticial.[7-9] São igualmente aqui inseridas as ILD de causa conhecida, como são exemplo aquelas resultantes de toxicidade pulmonar induzida por fármacos ou secundárias a uma exposição ambiental/profissional.

No segundo grupo são inseridas as IIP, que no recente consenso ERS/ATS publicado em 2013 são divididas em IIP major, IIP raras e IIP inclassificáveis.[10] Por sua vez as IIP major são divididas em IIP fibróticas crónicas, IIP associadas ao consumo tabágico e IIP agudas/subagudas (Tabela 1). As IIP major fibróticas crónicas englobam a IPF e a Pneumonia Intersticial Não Específica (NSIP) que se divide em formas celular e fibrosante. A IPF trata-se de uma doença associada a um processo patofisiológico fibrosante, em que após uma repetição de agressões ambientais de etiologia em grande parte desconhecida, ocorre uma resposta anómala na cicatrização, com deposição progressiva de matriz extracelular, condicionando uma evolução desfavorável, que se associa a uma sobrevida média entre 2-3 anos.[11-15]

Classificação	Diagnóstico Clínico - Radiológico - Histológico	Padrões morfológicos, histológicos e/ou radiológicos
IP fibróticos crónicos	Fibrose Pulmonar Idiopática Pneumonia Intersticial não específica	Pneumonia Intersticial Usual Pneumonia Intersticial não específica
IP relacionados com tabagismo	Bronquiolite Respiratória associada a ILD Pneumonia Intersticial Descamativa	Bronquiolite Respiratória Pneumonia Intersticial Descamativa
IP Aguda/Subaguda	Pneumonia Organizativa Criptogénica Pneumonia Intersticial Aguda	Pneumonia Organizativa Dano Alveolar Difuso

Tabela 1 Classificação das IIP Major. Adaptado da referência 10.

Dado o mau prognóstico, associado a uma abordagem terapêutica distinta das outras IIP, torna-se absolutamente determinante neste grupo um diagnóstico diferencial rigoroso que identifique e isole esta entidade de todas as outras que o compõe. [6, 10, 26]

O diagnóstico é baseado principalmente num quadro clínico caracterizado pela ocorrência de dispneia de esforço de evolução lenta e progressiva num indivíduo habitualmente com mais de 60 anos, que invariavelmente apresenta crepitações inspiratórias bibasais na auscultação pulmonar. A Tomografia Axial Computorizada com Cortes de Alta Resolução (HRCT), é o método de diagnóstico central nesta doença, dado que no caso de apresentar as alterações características que englobam a heterogeneidade espacial, o predomínio na periferia e bases pulmonares, com zonas de pulmão normal, intercaladas com zonas de espessamento interlobular prefigurando um padrão reticular e zonas com cistos em relação com um padrão em favo de mel, achados estes que no seu conjunto definem o padrão radiológico de Pneumonia Intersticial Usual (UIP), sustenta o diagnóstico (Figura 1).[11, 16-19]

No entanto, cerca de 1/3 dos casos de IPF não tem alterações radiológicas consistentes com UIP no contexto de uma IPF, devendo estes doentes, em caso de não terem contra-indicações, realizar uma biopsia pulmonar cirúrgica.[11, 16] Esta, no caso de evidenciar um padrão histológico de UIP e

Figura 1 Padrão radiológico de UIP em doente com IPF, com heterogeneidade que engloba zonas de pulmão normal, com zonas com padrão reticular e outras com padrão em favo de mel, predominando na periferia e nas bases de ambos os pulmões.

sempre no contexto da avaliação multidisciplinar que engloba as alterações radiológicas e a investigação clínica, poderá apoiar o estabelecimento do diagnóstico de IPF.[11, 16] No que concerne à NSIP, entidade descrita e proposta por *Katzenstein* e *Fiorelli* em 1994,[20] é na sua vertente fibrosante, mais usual, uma doença crónica mas com um prognóstico mais favorável que a IPF, associando-se a uma sobrevida de 80% após 5 anos decorridos do diagnóstico.[21, 22] Apesar de alterações imagiológicas características, torna-se por vezes difícil a sua diferenciação com o padrão UIP, nomeadamente na sua associação com a IPF, sendo habitualmente necessária a biopsia pulmonar cirúrgica para o seu diagnóstico diferencial.[22, 23]

Embora ocorram raramente formas idiopáticas, a NSIP apresenta-se mais frequentemente de forma secundária no contexto principalmente de conetivites, mas também associada a HP ou a toxicidade induzida por fármacos.[9, 10, 24] A forma celular de NSIP é rara e associa-se a um bom prognóstico, podendo ter inclusive resolução espontânea.[6, 10, 24, 25] As IIP associadas ao consumo tabágico englobam a Bronquiolite Respiratória com Doença Intersticial Associada (RB-ILD) e a Pneumonite Intersticial Descamativa (DIP).[26-28] A RB-ILD é um processo inflamatório que se traduz imagiologicamente pela presença de nódulos centrilobulares com padrão em vidro despolido, de forma mais significativa nos lobos superiores, sendo habitualmente um achado imagiológico, dado que se acompanha por ausência ou sintomas respiratórios discretos e igualmente por ausência ou alterações ligeiras no estudo funcional respiratório.[29, 30] A DIP, que aparentemente poderá resultar de uma evolução da RB-ILD, é uma doença com sintomatologia mais relevante, nomeadamente com dispneia de esforço, alterações funcionais mais marcadas, com síndrome ventilatório restritivo e diminuição da capacidade de difusão, para além de uma maior extensão do padrão imagiológico em vidro despolido, que para além de mais difuso, curiosamente é mais evidente nos lobos inferiores.[30-32]

Esta entidade exige uma confirmação por biopsia pulmonar, habitualmente cirúrgica, para que o seu diagnóstico seja estabelecido, existindo a indicação para tratamento com corticoide, e apesar do bom prognóstico global, ocorrem alguns casos com evolução desfavorável.[10, 28, 32] No grupo das IIP subagudas/agudas engloba-se a Pneumonia Organizativa (OP), processo inflamatório que se caracteriza pela presença de tufos de tecido de granulação no lúmen dos espaços aéreos distais e, por vezes no lúmen bronquiolar, encontrando-se associada a contextos variados e distintos

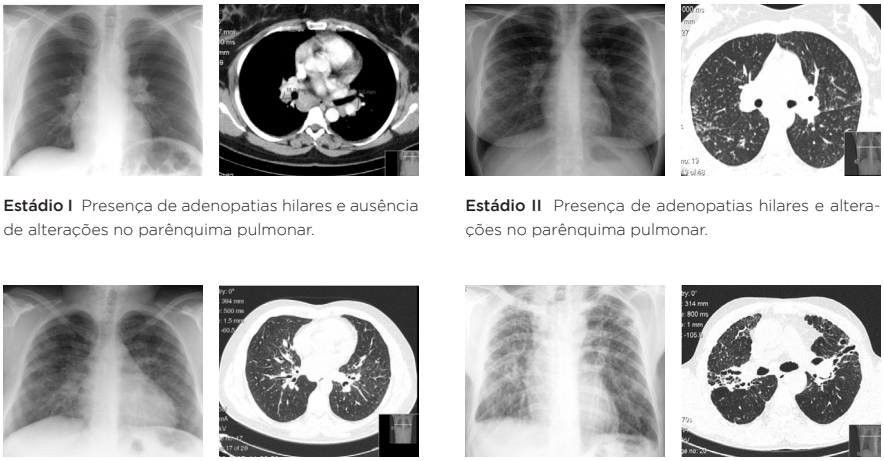
como infecções, toxicidade induzida por fármacos, imunodeficiências, doenças hematológicas, reacção enxerto *versus* hospedeiro ou doenças do tecido conjuntivo, podendo no entanto ser idiopática.[10, 25, 33, 34] Embora podendo apresentar recidivas, associa-se a um bom prognóstico, com resolução após tratamento prolongado com corticóide.[33, 35, 36] Ao contrário, a Pneumonia Intersticial Aguda (AIP), entidade rara, com uma forma de apresentação súbita e dramática, traduzida habitualmente numa insuficiência respiratória grave e necessidade frequente de assistência ventilatória invasiva, apresenta um mau prognóstico, verificando-se a morte intra-hospitalar da maioria dos doentes.[6, 10, 25]

O grupo das IIP raras engloba duas entidades, a Fibroelastose Pleuro-parenquimatosa Idiopática (PPFE) e a Pneumonia Intersticial Linfocítica Idiopática (LIP).[10] A PPFE foi descrita apenas em 2004 por *Frankel et al*, caracterizando-se por uma fibrose pleural e do parênquima subpleural de forma mais significativa nos lobos superiores.[37] A sua etiologia é desconhecida, sendo que para além das formas idiopáticas pode estar em contexto de outras patologias, como as doenças do tecido conjuntivo ou serem secundárias a toxicidade pulmonar por drogas.[38-42] Por outro lado, encontram-se frequentemente associadas a outras IIP como a UIP ou a NSIP.[39, 40] A LIP é extremamente rara como forma idiopática, sendo habitualmente observada num contexto secundário no âmbito da síndrome de *Sjögren*.[6, 10, 25]

As IIP inclassificáveis são consideradas sempre que existem dados clínicos imagiológicos ou histológicos inconclusivos, discordância entre o quadro clínico, alterações imagiológicas e histológicas ou na presença de eventual entidade ainda não adequadamente caracterizada.[10, 43, 44]

O 3º grupo inclui as doenças difusas associadas a inflamação granulomatosa, que englobam a sarcoidose e a HP, sendo estas as ILD mais frequentes.[6] A sarcoidose é uma doença multissistémica, que se caracteriza pela presença de uma linfandenite granulomatosa nos órgãos envolvidos.[45-48] A sua causa mantém-se desconhecida, sendo a ocorrência da doença provavelmente devida à exposição a agente(s) do meio ambiente, ainda por isolar, por parte de um indivíduo com predisposição genética para a doença.[49-51] Embora com uma distribuição global, a sarcoidose apresenta uma incidência e uma apresentação clínica variáveis segundo diferentes raças e áreas geográficas, sendo mais prevalente nos países nórdicos e nos Afro-Americanos.[45, 46, 52, 53] Os dados existentes, baseados no entanto em estudos com metodologia variável, sugerem uma incidência da doença de 10/100000 nos países da Europa Ocidental, e uma prevalência entre 10-20/100000, com um predomínio entre os 20 e os 40 anos e no sexo feminino.[45, 46] A apresentação clínica da sarcoidose é variada, dependendo nomeadamente do local e extensão do(s) órgão(s) envolvidos e da intensidade da inflamação granulomatosa. Dado o pulmão ser o órgão mais frequentemente envolvido, ao atingir mais de 90% dos doentes, os sintomas respiratórios como a dispneia e a tosse são referidos

por cerca de 20-50% dos doentes, ocorrendo sintomas constitucionais em cerca de 1/3.[45, 53-55] A síndrome de *Löfgren* constituída por eritema nodoso, poliatralgias e adenopatias hilares bilaterais simétricas, de início agudo, atinge geralmente jovens do sexo feminino, sendo uma das formas de apresentação mais comuns, ocorrendo em cerca de 20% dos doentes originários do Norte de Portugal.[45, 52, 56] Na abordagem diagnóstica desta doença, para além da história clínica e do exame físico, que podem sugerir quer o diagnóstico quer o tipo de envolvimento orgânico, o estudo imagiológico, nomeadamente torácico, é primordial, dadas as alterações características habitualmente observadas. As manifestações radiográficas da sarcoidose são classificadas segundo o critério de *Scadding* em quatro grupos ou estadios: estadio I: adenopatias mediastino-hilares e ausência de envolvimento pulmonar; estadio II: adenopatias mediastino-hilares e envolvimento pulmonar; estadio III: ausência de adenopatias mediastino-hilares e envolvimento pulmonar; estadio IV: envolvimento pulmonar com fibrose (Figura 2).[57]



Estádio I Presença de adenopatias hilares e ausência de alterações no parênquima pulmonar.

Estádio II Presença de adenopatias hilares e alterações no parênquima pulmonar.

Estádio III Doença pulmonar difusa e ausência de adenopatias.

Estádio IV Presença de fibrose no parênquima pulmonar.

Figura 2 Estádios radiológicos do envolvimento torácico pela sarcoidose segundo os critérios de *Scadding*.

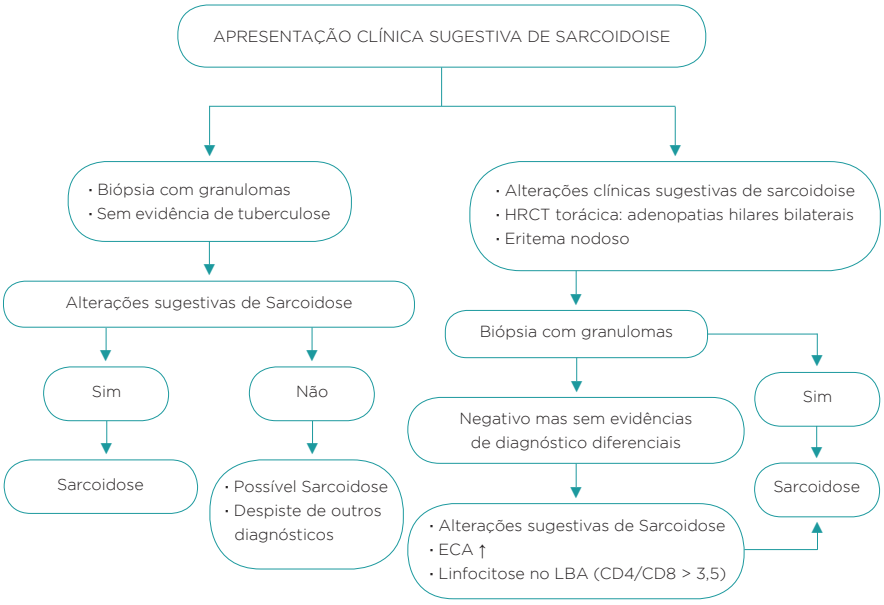
A combinação de adenomegalias hilares bilaterais simétricas e paratraqueais direitas é uma manifestação característica comum, estando as primeiras presentes em 95% e as segundas em 70% na radiografia torácica dos doentes, estando obviamente estes valores aumentados na HRCT, por esta mostrar um maior número de compartimentos mediastínicos.[58-61] Como é de esperar, as alterações pulmonares são igualmente mais frequentemente observadas na HRCT do que na radiografia pulmonar, demonstrando esta a distribuição característica dos granulomas ao longo do interstício dos feixes broncovasculares, dos espaços subpleurais e perilobulares.[58-61]

O padrão mais frequente e característico é o padrão nodular, evidenciando habitualmente nódulos de contornos irregulares e dimensões variáveis, entre 1 a 10 mm de diâmetro, embora a maioria apresente dimensões em volta dos 3 mm, com uma distribuição predominante nos lobos superiores que quando coexistente com adenomegalias mediastínicas sugere fortemente o diagnóstico de sarcoidose.[58-61]

Dado o aumento da sensibilidade, quer na detecção de alterações mediastínicas e pulmonares assim como na caracterização das mesmas, a HRCT torácica parece ser o exame de eleição no estudo de doentes com suspeita de sarcoidose. No entanto este exame é apenas considerado em determinadas situações, nomeadamente no consenso ATS/ERS/WASOG: presença de imagens atípicas na radiografia torácica, alterações funcionais ou suspeita clínica com radiografia torácica normal, estudo de sintomas pouco habituais como hemoptises ou intercorrências (infecção ou malignidade), suspeita de complicações como bronquiectasias, aspergiloma ou enfisema de tracção, estudo para orientação de biopsia transbrônquica ou doença do tracto respiratório superior.[45] Apesar das alterações características observadas na HRCT da maioria dos doentes, a confirmação ou maior sustentação diagnóstica exige habitualmente a realização de técnicas endoscópicas, durante as quais são realizadas técnicas de biopsia - brônquica e transbrônquica - e Lavado Broncoalveolar (BAL), nomeadamente para contagem celular total e diferencial, para além do estudo fenotípico das subpopulações linfocitárias.[45, 47] Efetivamente, a presença de linfocitose com uma relação CD4/CD8> 3,5, associa-se a uma especificidade de 92,5%, o que leva a ser considerado suficiente para o estabelecimento do diagnóstico de sarcoidose em casos com apresentação clínica e imagiológica sugestiva.[45, 62-64]

A biópsia brônquica tem uma sensibilidade diagnóstica de 40-60%, sendo mais elevada na presença de alterações endoscópicas, podendo a sensibilidade nestes casos alcançar os 90%.[45, 46, 65] Quando presentes as lesões endobrônquicas sugestivas de sarcoidose, apresentam caracteristicamente uma elevação nodular da mucosa brônquica com cerca de 2 a 3 mm, manifestando geralmente um aspecto granitado. A biopsia transbrônquica (BTB) apresenta igualmente uma elevada sensibilidade (40-90%) na obtenção de amostras de tecido pulmonar com alterações histológicas compatíveis com o diagnóstico de sarcoidose.[45, 46, 65-67] Esta sensibilidade aumenta com o número de colheitas de tecido pulmonar efetuadas, preconizando-se como desejáveis 4 a 6 biopsias e com um maior envolvimento pulmonar (estádios II-IV). Mais recentemente a generalização da utilização da Biopsia Ganglionar Transbrônquica por Ecoendoscopia Endobrônquica (EBUS-TBNA), levou a que seja um meio de diagnóstico utilizado com frequência crescente no contexto de sarcoidose com adenopatias mediastínicas, estando reportada uma sensibilidade diagnóstica entre 70-95%.[68-70] O diagnóstico de sarcoidose deverá ser idealmente suportado por confirmação histológica.[71, 72] No estudo inicial, deverão ser avaliados locais de eventual envolvimento pela doença que permitam um

acesso fácil para a realização de biopsia, como por exemplo a pele, gânglios periféricos, conjuntiva palpebral, saco lacrimal, nódulos subcutâneos, parótida ou mucosa nasal.[47, 71] Entretanto, o diagnóstico de sarcoidose será sempre um diagnóstico de exclusão, uma vez que não existe nenhuma alteração clínica, imagiológica ou histológica considerada patognomónica da doença (Quadro 2).[47, 67, 71] Após o diagnóstico de sarcoidose estar estabelecido, o objectivo deverá ser orientado na determinação da extensão do envolvimento e da gravidade da doença.[45, 47, 53, 73, 74] Dado o envolvimento torácico se verificar na quase totalidade dos doentes com sarcoidose, o estudo efetuado na altura do diagnóstico é dirigido preferencialmente para este segmento corporal.[48, 66, 71]



Quadro 2 Organigrama do diagnóstico da sarcoidose. Adaptado de referência 45.

No entanto, todos os órgãos podem ser envolvidos pela sarcoidose, sendo que a frequência reportada é muito distinta para cada órgão, sendo igualmente variável segundo a origem geográfica.[45, 73, 74] Num estudo efetuado em doentes com sarcoidose originários do Norte de Portugal, foi reportado envolvimento extra-torácico em 51,1% dos doentes, sendo o envolvimento cutâneo em 19,3%, ocular em 13,1% e hepático em 9,9%, os orgãos mais frequentemente atingidos. A sarcoidose apresenta em grande parte um bom prognóstico, com cerca de 2/3 dos doentes a apresentarem regressão da doença, em grande parte de forma espontânea. Nos doentes que evoluem para formas crónicas, verifica-se uma grande heterogeneidade, desde uma doença estável sem necessidade de intervenção terapêutica, até formas associadas a fibrose pulmonar e insuficiência respiratória com necessidade de intervenção terapêutica permanente.[45, 75-78]

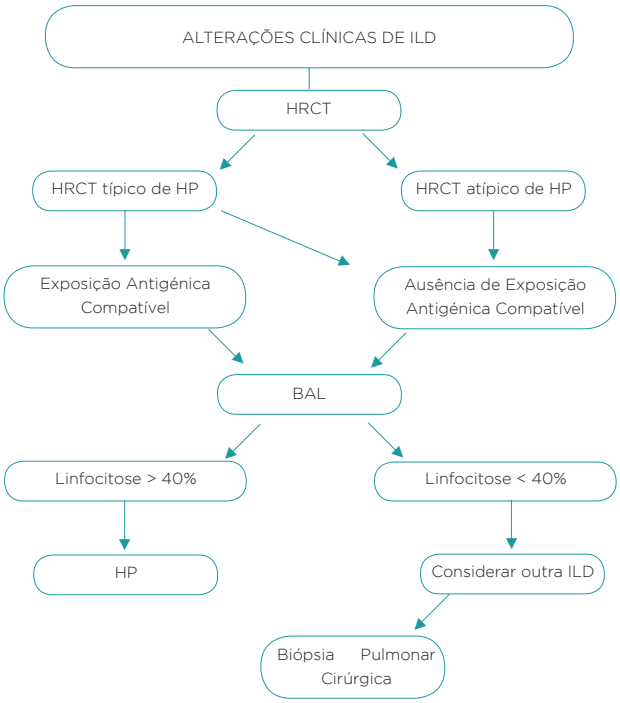
A HP é uma doença causada pela exposição a uma variedade de antigénios (Tabela 2) maioritariamente de natureza orgânica, de pequena dimensão (5 um), de forma a conseguirem alcançar os alvéolos, em indivíduos suscetíveis, causando uma resposta imune exagerada a nível dos bronquíolos e parênquima pulmonar.[79-82] Dado que num grupo de indivíduos com a mesma exposição antigénica, apenas uma pequena percentagem vem a desenvolver a doença, a presença de polimorfismos genéticos predisponentes, eventualmente acompanhados de fatores de ordem ambiental que contribuam para essa mesma suscetibilidade é a hipótese mais considerada.[79-82] Existe uma variedade significativa de antigénios desencadeantes da doença segundo as diferentes áreas geográficas que são dependentes de causas laborais, ambientais, ou até lúdicas, sendo as proteínas aviárias, fungos relacionados com indústria corticeira e fungos em áreas húmidas com formação de bolor, os agentes mais frequentemente relatados como causa desta doença em Portugal.[79, 83] Relativamente à suscetibilidade genética do hospedeiro, os dados disponíveis são significativamente em menor número quando comparados com a sarcoidose, sendo no entanto os alelos da classe II do HLA os mais referidos.[79, 84-86] Existem outras referências a polimorfismos relacionados com os transportadores associados ao Processamento de Antigénios (TAP) ou de citocinas como o TNF-α.[85, 87, 88] Relativamente ao processo fisiopatológico na resposta ao antigénio, verifica-se inicialmente uma reação de hipersensibilidade III, de tipo humoral, com formação de imunocomplexos, seguida de uma reação de hipersensibilidade IV, de tipo celular.[79,80]

Pulmão do Fazendeiro	*Thermophilic actinomycetes* *Saccharopolyspora rectivirgula*
Bagaçose	*Thermophilic actinomycetes*
Fabricação de queijos	*Penicillium sp*
Frequentadores de saunas	*Pullularia sp*
Trabalhadores em ambiente com ar condicionado e/ou umidificado	*Thermophilic actinomycetes*
Fabricação de detergentes	*Bacillus subtilis*
Indústria de alimentos	*Bacillus subtilis* e enzimas
Granjeiros	Enzimas proteolíticas (caseina)
Trabalho em biotério	Fezes de Aves Urina de rato macho
Suberose	Cortiça mofada (*Penicillium frequentans*)
Sulfato de cobre (vinícola)	Fungicida
Pneumonite "summer type"	*Trichosporon Cutaneum*
Pulmão dos trabalhadores de cogumelos	*Trichosporon* sacchari
Pulmão dos trabalhadores de malte	*Aspergillus clavatus*
Hot Tub Lung	*Mycobacterium Avium*
Pulmão dos trabalhadores de saunas	*Aureobasidium Graphium spp*

Tabela 2 Antigénicos causadores de pneumonite de hipersensibilidade.

Define-se habitualmente a apresentação clínica da HP em aguda, subaguda e crónica.[79] A apresentação aguda verifica-se quando o doente apresenta sintomas semelhantes a uma síndrome gripal, com febre, tosse seca, mialgias, fadiga, pieira, dispneia, com início 4 a 8 horas após o início da exposição, o pico de intensidade entre as 12 e 24 horas terminando 48 horas após a cessação da exposição.[79, 81, 89] A apresentação subaguda, resulta da exposição repetida a baixas doses de antigénio, caracterizando-se por dispneia de esforço, fadiga e tosse episódica com duração de semanas a alguns meses.[79, 81, 89] Dos três tipos de apresentação é a mais difícil de caracterizar e é um dos motivos de contestação desta classificação.[89, 90] A apresentação crónica desenvolve-se de forma insidiosa durante meses ou anos em indivíduos com frequente ou contínua exposição antigénica, podendo desenvolver fibrose pulmonar, com quadros clínicos e imagiológicos idênticos às pneumonias intersticiais crónicas, incluindo padrão radiológico de UIP e NSIP.[79, 81, 91] O diagnóstico é baseado na apresentação clínica num doente com exposição compatível com a doença, sustentado pelas alterações habitualmente sugestivas na HRCT.[79, 89] Este apresenta nódulos centrilobulares em vidro despolido principalmente na apresentação aguda, observando-se um padrão característico em mosaico, por vezes em alternância a um padrão em vidro despolido, formando o sinal *head cheese* nomeadamente na apresentação subaguda.[92, 93] Nas formas crónicas, este sinal pode ser acompanhado por fibrose, inclusive com um padrão de UIP ou NSIP de tipo fibrosante, podendo a HRCT mostrar alterações idênticas aquelas expressas pelas pneumonias intersticiais crónicas fibrosantes.[92, 94, 95]

Para além das alterações características que a HRCT apresenta na maioria dos casos de HP, os doentes são frequentemente orientados para a realização de BAL, na qual se observa habitualmente uma linfocitose intensa (> 40%) e embora com assinalável variabilidade, um predomínio de células $CD8^+$.[83, 96-98] Embora não seja de todo um valor patognomónico, verifica-se, no entanto, que nas outras doenças que cursam com linfocitose, como na sarcoidose, esta é habitualmente ligeira a moderada, embora alguns casos de pneumonia organizativa possam cursar igualmente com linfocitose intensa.[62] Quando temos presente uma história clínica compatível, nomeadamente com exposição associada a HP, conjuntamente com alterações imagiológicas características na HRCT e linfocitose intensa no BAL, o diagnóstico de HP pode ser estabelecido sem necessidade de biopsia pulmonar.[79, 81] Nos casos em que estes achados não estão presentes, o doente deverá ser orientado para biopsia pulmonar, estando descrito que cerca de 30% dos casos necessitem desta técnica.[89, 91] Habitualmente, tratam-se de casos de HP crónica, em que as alterações imagiológicas podem ser semelhantes nomeadamente às pneumonias intersticiais crónicas, como referido anteriormente, adicionado ao fato de serem frequentemente os casos crónicos, com fibrose, aqueles em que se verifica uma menor intensidade da linfocitose ou mesmo ausência da mesma no BAL.[79, 91, 95]



Quadro 3 Organigrama de diagnóstico da pneumonite de hipersensibilidade. Adaptado da referência 79.

No 4º grupo encontram-se um conjunto de doenças raras e heterogéneas, nomeadamente as doenças císticas e a proteinose alveolar.[6] As doenças císticas englobam a Histiocitose Pulmonar de Células de *Langerhans* (PLCH) e a Linfangioleiomiomatose (LAM) - esporádica e associada a esclerose tuberosa.[99-101] A PLCH faz parte de um conjunto variado de patologias de causa desconhecida que têm como ponto comum apresentarem uma proliferação exagerada de células de *Langerhans*.[102, 103] Ocorre geralmente em adultos jovens, envolvendo apenas ou de forma predominante o pulmão. [104, 105] Na HRCT são visíveis nos campos superiores e médios pulmonares nódulos, nódulos cavitados, cistos de parede grossa e cistos de parede fina, parecendo ser esta a ordem de evolução das lesões, correspondendo cada uma delas a diferentes fases da doença.[106, 107] Em relação ao envolvimento extra-pulmonar, as lesões ósseas, da pele e o atingimento da hipófise com diabetes insípida são as alterações mais frequentemente observadas no adulto.[105] É uma doença relacionada com o consumo tabágico, uma vez que cerca de 90 a 100% dos doentes são fumadores.[103-105] A sua evolução é variável, podendo regredir completamente, estabilizar ou evoluir para a insuficiência respiratória, sem que, até ao momento, tenhamos meios terapêuticos que influenciem o curso da doença.[103, 104] A LAM afeta quase exclusivamente mulheres, habitualmente em idade fértil.[108-110] A doença caracteriza-se pela proliferação de células atípicas (células de LAM) que exibem características de neoplasia de baixo grau, com potencial metastático, nos pulmões e nos linfáticos

torácicos e retroperitoniais.[108-110] Clinicamente caracteriza-se por dispneia de esforço, tosse seca, episódios de pneumotórax e menos frequentemente quilotórax.[108-110] A HRCT mostra a presença de cistos de parede fina, mais regulares que na PLCH e dispersos de forma difusa.[111] Após um longo período em que o único tratamento modificador da evolução da doença era o transplante pulmonar, atualmente os inibidores da *mammalian target of rapamycin* (mTOR), como o sirolimus ou o everolimus têm mostrado eficácia quer a nível das alterações pulmonares quer nas alterações extra-torácicas, condicionando um prognóstico significativamente mais favorável.[109, 112]

A proteinose alveolar pulmonar é uma doença extremamente rara, com uma prevalência calculada de 3,7/milhão, predomínio masculino, sendo 80% dos casos diagnosticados entre os 30 e 40 anos.[113-115] Engloba três formas clínicas: congénitas, adquiridas e secundárias.[113] Caracteriza-se por uma acumulação alveolar de abundante material fosfolipoproteináceo, resultante dos produtos de degradação do surfactante, que não são digeridos pelos macrófagos, dada a sua função anómala resultante de uma deficiência no fator de crescimento (GM-CSF).[113] A terapêutica de eleição mantém-se a lavagem pulmonar total, sendo variável o número de lavagens necessárias em cada doente, mantendo-se no entanto uma terapêutica eficaz no controle da doença na maior parte dos doentes.[113-115]

ABORDAGEM DIAGNÓSTICA DAS ILDS

A apresentação clínica destas doenças é habitualmente pouco diversificada, apresentando os doentes invariavelmente dispneia de esforço e tosse, geralmente não produtiva.[1-4] No entanto, uma história clínica completa é de extrema importância para a orientação diagnóstica, a começar pelos elementos de identificação como a idade (a sarcoidose associa-se maioritariamente a idades jovens, ao contrário por exemplo da IPF que ocorre geralmente em indivíduos com mais de 50 anos) ou o sexo (a ocorrência quase exclusiva da linfangioleiomiomatose no sexo feminino).[1-5]

A história do consumo tabágico pode-nos orientar para patologias com associação a esta exposição como a DIP, a RB-ILD ou a PLCH.[1-5] A exposição profissional e a medicação efectuada são outros dos pontos a ter em atenção, dado que podem apresentar-se como a etiologia da doença em estudo. Em relação à exposição, que pode ou não ser profissional, destacam-se as que podem induzir a HP, nomeadamente a cortiça e as aves.[1-5, 25] Adicionalmente o exame físico que frequentemente não nos dá informações abundantes, sendo as crepitações inspiratórias a alteração mais comum na auscultação pulmonar, podendo ainda verificar-se uma diminuição dos movimentos e dos sons respiratórios nos doentes que já apresentam uma restrição torácica importante.[1-5] Igualmente podem ser visíveis alterações que nos sugiram patologias de envolvimento sistémico como por exemplo as alterações cutâneas observadas na esclerose

sistémica ou as alterações osteoarticulares associadas à artrite reumatóide. Existem também alguns sinais clínicos, que no contexto destas doenças se associam a algumas das entidades como é o exemplo do eritema nodoso e a sarcoidose ou os dedos em baqueta de tambor e a IPF. [1-5, 25]

Analiticamente são de extrema importância os marcadores de investigação das doenças do tecido conjuntivo como os anticorpos antinucleares e anticorpos anti-ENA, dado o envolvimento pulmonar, nomeadamente intersticial, ser frequente neste contexto.[7, 8, 116, 117] Igualmente importante é a pesquisa de anticorpos anti-citoplasma dos neutrófilos, no despiste de uma eventual vasculite, nomeadamente na existência de um quadro clínico sugestivo de hemorragia pulmonar.[118] A Enzima de Conversão da Angiotensina é pedida no contexto de suspeita de sarcoidose, apresentando uma sensibilidade de 75%, embora associada a uma baixa especificidade, o que questiona a sua relevância no diagnóstico da doença.[45, 119] Em caso de suspeita de HP em doentes com determinado tipo de exposição, são pedidas precipitinas, que são anticorpos IgG contra antigénios passíveis de causar esta doença, como são exemplo as precipitinas aviárias. No entanto, para além de uma padronização laboratorial ainda não totalmente validada, as precipitinas podem ser positivas em indivíduos expostos e não doentes, sendo assim mais marcadores de exposição, para além de em caso de estarem ausentes não eliminam o diagnóstico da doença, o que relativiza de forma muito significativa o seu papel diagnóstico.[25, 79]

O estudo funcional respiratório é igualmente imprescindível no estudo das ILD, permitindo-nos observar nomeadamente o tipo de alteração ventilatória presente, para além da gravidade da mesma. A maior parte das doenças que compõem este grupo cursa com uma alteração ventilatória restritiva, quando apresentam um envolvimento intersticial importante que condiciona uma diminuição da distensão torácica. Para além da avaliação dos volumes pulmonares, pelo tipo de envolvimento predominante nestas doenças, é fundamental o estudo da transferência alvéolo-capilar, sendo por vezes a única alteração presente. Realce-se no entanto que alguns doentes não apresentam qualquer anormalidade funcional numa fase inicial da doença. Dado o envolvimento intersticial, um estudo funcional completo necessita da avaliação do esforço, nomeadamente através da prova da marcha dos 6 minutos e da prova de esforço cardiorespiratória. [1-5, 25, 120]

A avaliação radiológica é determinante na abordagem diagnóstica das ILD, sendo um dos pilares desse mesmo diagnóstico. Geralmente, esta avaliação inicia-se por uma radiografia torácica que na maior parte das vezes mostrará um padrão reticular difuso, que pode no entanto ser ténue e não valorizado. A HRCT torácica com cortes de alta resolução para análise do parênquima pulmonar torna-se incontornável na quase totalidade dos casos pelo(s) padrão(ões) radiológico(s) exibido(s) (ex: vidro despolido, mosaico, nodular, cistos, favo de mel...), pela sua distribuição (peribroncovascular, periférica, basal...), para além da gravidade evidenciada, nomeadamente através da extensão das alterações observadas ou da pre-

sença de destruição da arquitetura normal do pulmão. O conjunto de todas estas alterações apresentam uma sensibilidade e especificidade elevadas em relação ao diagnóstico final, permitindo assim uma orientação diagnóstica com elevado grau de fiabilidade. Para além da análise do parênquima pulmonar, a avaliação do mediastino torna-se importante uma vez que a presença de adenopatias pode sugerir o diagnóstico de sarcoidose, sendo que a radiografia torácica neste caso poderá ser esclarecedora ao mostrar imagens sugestivas de adenopatias mediastínicas e hilares, que serão obviamente mais evidentes na HRCT torácica. [6, 11, 25, 45, 120]

Das técnicas invasivas de diagnóstico, aquelas realizadas através da broncofibroscopia, merecem um destaque central. A avaliação endoscópica respiratória é um dos procedimentos imprescindíveis na abordagem destes doentes, sendo a sua importância resultante não só da visualização de eventuais alterações da traqueia e árvores brônquicas, como por ser a via de realização do BAL, biopsias brônquicas, biopsia ganglionar mediastinica transbrônquica, bem como da biopsia pulmonar transbrônquica (BTB).[25] Em primeiro lugar, a avaliação endoscópica permite a detecção de alterações endobrônquicas, que ao serem biopsadas poderão em si mesmo fornecer a sustentação do diagnóstico, como é exemplo o envolvimento brônquico pela sarcoidose.[45] Por outro lado a realização do BAL permite o estudo das células do compartimento alveolar, podendo obter-se informações que podem ser decisivas para o diagnóstico.[45, 62, 63, 121-125] Esta técnica é efetuada após encravamento do broncofibroscópio, geralmente nos brônquios subsegmentares do brônquio lobar médio, com instilação de pelo menos 150-300 ml de soro fisiológico dividido em várias seringas com posterior aspiração.[62, 125] O volume recuperado é de seguida processado em laboratório, sendo inicialmente filtrado por uma gaze estéril. A contagem celular total é efetuada numa câmara de *Neubauer* a partir de uma diluição do produto homogeneizado em violeta de cristal, sendo os resultados expressos em número de células contadas por mililitro (nº células $x10^4/ml^{-1}$). A contagem celular diferencial é realizada em 2 esfregaços de citocentrífuga, de 40 Ql e 120 Ql do produto homogeneizado, a 1000 rpm., 30 segundos e corados com *Wright/Giemsa*, sendo contadas e analisadas 500 células (Figura 3). Poderão ser efetuadas várias colorações adicionais, como são exemplo os casos da coloração de *Pearls* na suspeita de hemorragia, para a deteção de hemossiderina no citoplasma dos macrófagos ou a coloração PAS na suspeita da presença de material fosfolipídeo ou ainda a coloração de Negro do Sudão ou OilRed na deteção de inclusões lipídicas no citoplasma dos macrófagos.[62, 63, 121, 122, 126] O estudo de fenotipagem celular, nomeadamente dos linfócitos é efetuado em citometria de fluxo com anticorpos monoclonais .[62]

Relativamente ao seu papel no diagnóstico, o BAL pode em determinadas situações ser decisivo, como no caso da presença de> 5% de células $CD1a^+$ na PLCH, na observação de material PAS^+ resultante da presença de fosfolipoproteinas derivadas do surfactante na proteinose alveolar, na

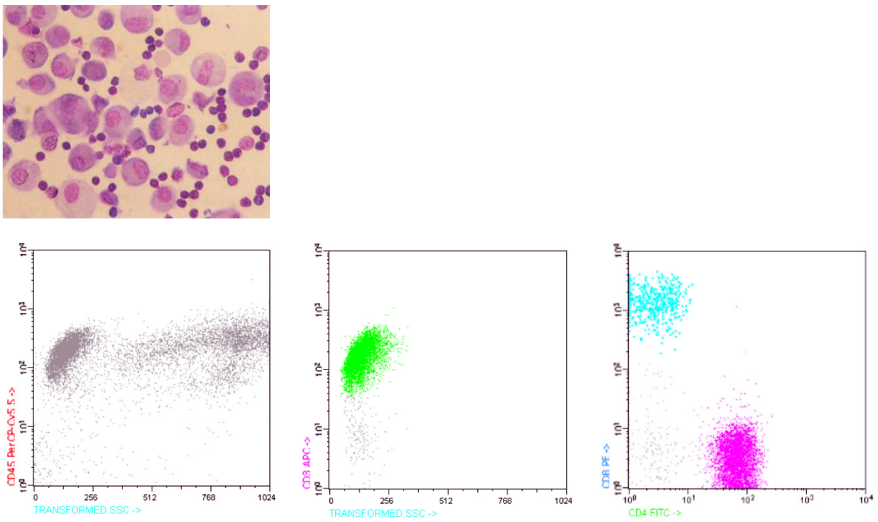


Figura 3 Contagem celular diferencial com linfocitose e imunofenotipagem por citometria de fluxo onde é evidente um predomínio CD4+, no BAL de um doente com sarcoidose.

detecção de hemossiderina no citoplasma dos macrófagos resultante de hemorragia alveolar ou na presença de >20% de eosinófilos na contagem celular diferencial tradutor de uma síndrome eosinofílica.[62, 63, 127, 128] Noutras situações, o BAL pode num contexto clínico e radiológico característico, sustentar o diagnóstico e obviar à necessidade de obtenção de biopsia pulmonar para caracterização histológica, como é o caso das doenças difusas granulomatosas.[62, 63, 71, 97, 125, 128] Na sarcoidose, encontra-se estabelecido há mais de duas décadas uma especificidade elevada, de cerca de 94% nos doentes com uma relação CD4/CD8> 3,5, pese embora apenas cerca de 54% apresentem este valor.[45, 62-64, 71, 125, 128] Por outro lado, na HP, no contexto de uma exposição compatível e de alterações imagiológicas características, a presença de uma linfocitose intensa (>35%), independentemente do valor da relação CD4/CD8 que é variável, sustenta igualmente o diagnóstico, sem necessidade de procedimentos adicionais.[13, 62, 97, 126, 128] Noutras entidades, como é exemplo a pneumonia organizativa, a ocorrência de consolidações de predomínio periférico, por vezes de caracter migratório adicionado à presença de linfocitose, igualmente intensa, é considerado suficiente para o estabelecimento daquele diagnóstico.[62, 63, 121] Contrariamente, na pneumonia intersticial usual e na pneumonia intersticial não específica de tipo fibrosante, o BAL habitualmente não distingue não só estas duas entidades, como os diversos contextos em que se manifestam, ao serem observadas maioritariamente neutrofilia associada a eosinofilia.[10, 16, 25, 122, 128] Num número pequeno de casos, no entanto, a presença de linfocitose, por vezes intensa, permite a orientação para um diagnóstico diferente da IPF, como por exemplo a UIP no contexto de uma PH, dado que aquela raramente apresenta linfocitose e quando presente é ligeira.[98] Se este pequeno número de casos em que o BAL poderá ser determinante na abordagem diagnóstica é suficiente para a realização por rotina deste procedimento nos casos de UIP ou NSIP fibrosante mantém-se uma questão em aberto.[11, 16]

Apesar de ter sido idealizada e ser utilizada preferencialmente no contexto do estadiamento do cancro do pulmão, a biopsia ganglionar transbrônquica guiada por ecoendoscopia (EBUS-TBNA) é efetuada no diagnóstico de adenopatias mediastínicas, independentemente da sua etiologia.[70] A sua utilização no contexto das doenças que temos vindo a comentar, assenta sobretudo nos casos de estadios I e II da sarcoidose, em que podemos obter o diagnóstico através da análise de material obtido das adenopatias acessíveis a punção transbrônquica, sendo um procedimento com elevada acuidade diagnóstica e de utilização crescente na abordagem diagnóstica da sarcoidose.[68-70]

A biopsia transbrônquica convencional, efetuada por broncofibroscopia permite a obtenção de tecido pulmonar, devendo no mínimo ser retiradas 4 amostras.[129, 130] Embora se possa obter o diagnóstico de várias entidades, como a HP, OP, pneumonia eosinofílica ou PLCH, este procedimento tem a sua importância essencialmente na sarcoidose, onde apresenta uma sensibilidade elevada (40-90%).[25, 129, 131-133] Em todas as outras situações, a sensibilidade é diminuta, sendo que noutras entidades como a das pneumonites intersticiais, exceptuando a OP, é impossível obter o diagnóstico com esta técnica.[10, 11, 25, 129, 134] Esta situação deve-se ao facto da dimensão das amostras colhidas ser diminuta, não permitindo obter tecido pulmonar suficiente para estabelecer o padrão histológico de forma conclusiva.[10, 11, 25, 131] Esta pequena dimensão deve levar a interpretar-se com cautela as alterações observadas, dado poderem ser apenas uma pequena amostra da lesão observada. Um exemplo é o da OP que pode ser o padrão representativo da lesão ou ser apenas um dos componentes histológicos observados na periferia das mesmas, nomeadamente nos casos de etiologia infeciosa ou neoplásica.[33] Para além da dimensão das amostras colhidas ser diminuida (média de 1,4 x 1 mm), outro dos motivos da baixa sensibilidade diagnóstica é a presença de artefactos de esmagamento que contribuem para uma má qualidade da amostra obtida.[135-137] Esta situação levou ao desenvolvimento de técnicas que englobavam pinças de biopsia com possibilidade de obter amostras de maior dimensão, efectuadas habitualmente através de broncoscopia rígida.[136, 137] Mais recentemente, têm sido descrito por vários grupos a realização de criobiopsias transbrônquicas, que permitem uma representação de pulmão em maior número de casos, para além de uma significativa maior dimensão das amostras e melhor qualidade das mesmas, dada a ausência de artefactos de esmagamento.[138-140] O princípio do equipamento das criobiopsias relaciona-se com o efeito *Joule-Thomson*, que refere que um gás comprimido (no caso dióxido de carbono ou oxido nítrico), libertado sob alta pressão (45 bar), expande-se e cria uma temperatura muito baixa (80 a 89ºC).[135, 137] Este procedimento é efetuado através de broncoscopia, sendo o lobo e os segmentos pulmonares escolhidos após observação da HRCT torácica.[135] Dado o risco de hemorragia, é introduzido previamente à realização das criobiopsias transbrônquicas um cateter de *Fogarty* no segmento escolhido, após o que se introduz o broncofibroscópio e posteriormente a sonda de criobiopsia (1,9 ou 2,4 mm), sendo aconselhadas 3-6 biopsias (Figura 4).[135]

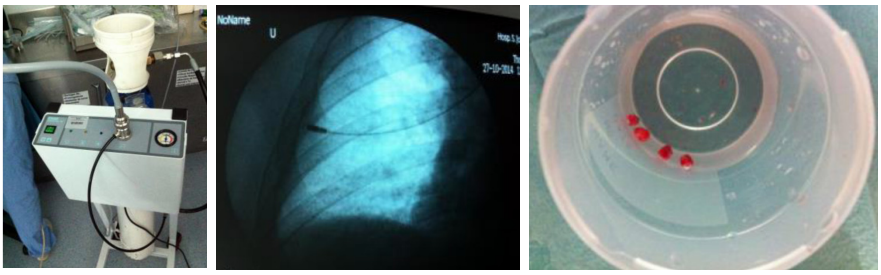


Figura 4 Material e realização de criobiopsias transbrônquicas

Esta técnica, tem permitido uma elevada representação pulmonar (99% *versus* 84% da biopsia transbrônquica convencional), tendo as biopsias uma dimensão, que embora com alguma variabilidade segundo as séries descritas, apresentam dimensões entre 4-9 mm.[135, 141] A acuidade diagnóstica é igualmente elevada, em cerca de 70%, incluindo a UIP e a NSIP cujo reconhecimento exigia até ao presente a realização de biopsia pulmonar cirúrgica.[135, 140, 141] Terá de ser considerado, no entanto, a presença de efeitos secundários significativos, nomeadamente com uma elevada incidência de pneumotórax, em algumas séries com registos entre 25-30%.[141, 142]

Finalmente, se após os procedimentos descritos, a avaliação multidisciplinar não concluir o diagnóstico, o doente deverá ser orientado para biopsia pulmonar cirúrgica, após avaliação da relação risco/ benefício.[10, 11, 25, 143, 144] Este procedimento permite obter fragmentos de tecido pulmonar de dimensão suficiente para uma avaliação que permita a obtenção do padrão histológico associado à doença em estudo. Este procedimento deverá ser efetuado preferencialmente por video-toracoscopia, método que na literatura publicada tem sido associado a menor morbilidade.[143, 145] Por outro lado, torna-se necessária a obtenção de amostras em mais de um lobo pulmonar por se ter observado a existência de diferentes padrões histológicos nas diferentes proveniências.[143, 144] O exemplo mais evidente é a presença de um padrão de UIP e NSIP em lobos diferentes do mesmo doente, o que tem implicações terapêuticas e de prognóstico, dado que neste caso deverá prevalecer o padrão UIP.[144] Reafirme-se no entanto, que o diagnóstico é sempre estabelecido após consenso na avaliação multidisciplinar, que deverá ter a colaboração além dos clínicos, de dois radiologistas e dois patologistas, devendo a biopsia pulmonar ser avaliada neste contexto.

IMUNOGENÉTICA E IMUNOPATOGENIA NAS ILDS

Como foi referido anteriormente, a sarcoidose é uma doença caracterizada por uma inflamação granulomatosa com envolvimento multissistémico.[46, 49, 52] O envolvimento torácico é o mais comum, afetando cerca de 90% dos doentes.[46, 49, 52] A sua causa não é conhecida, conjeturando-se que possa ser resultante da exposição a um agente ambiental por parte de um indivíduo com predisposição genética para desenvolver a doença,

determinada pela interação de um conjunto de polimorfismos genéticos, nomeadamente relacionados com a resposta imunitária.[146, 147] Alguns dos fatores que sugerem a predisposição genética são a ocorrência de agregação familiar, descrita com um risco relativo entre 5 - 10%, e a diferente prevalência, tipo de apresentação clínica e grau de gravidade associados a diferentes raças e regiões, como é exemplo a maior incidência e gravidade da doença nos negros americanos comparativamente com os caucasianos.[146, 147]

Existem vários estudos sobre polimorfismos genéticos e a sua associação com a suscetibilidade da doença, como por exemplo os relacionados com o sistema de Antigénios Leucocitarios Humanos (HLA) do complexo major de histocompatibilidade (MHC), com os genes das citocinas ou das quimocinas participantes na resposta imunológica ou com o gene da enzima de conversão da angiotensina sérica (SACE).[146-148] O sistema HLA, localizado no braço curto do cromossoma 6, participante na regulação da resposta imune, nomeadamente através do reconhecimento de antigénios, no seu processamento e na sua apresentação aos linfócitos $CD4^+$ através da sua classe II, ou aos linfócitos $CD8^+$ pela sua classe I, foi até agora o mais investigado, com a publicação de dados referentes a várias e distintas populações, sendo igualmente aquele com maior evidência na associação com a suscetibilidade da doença e com a sua variada expressão.[149-151] Num estudo efetuado numa população que englobou 104 indivíduos caucasianos originários da região norte de Portugal, com o diagnóstico de sarcoidose, em comparação com 124 controlos correspondentes a indivíduos saudáveis com a mesma origem, verificou-se relativamente às frequências alélicas do HLA dos doentes que os alelos *B*08* (OR=1,83), *DRB1*03* (OR=1,83) e *DRB1*12* (OR=2,63) eram aqueles que apresentavam uma maior associação com a doença, não se verificando no entanto significância estatística quando os seus valores de *p* eram corrigidos para as múltiplas comparações (Tabela 3).[152] As frequências dos alelos *B*44*, *B*51, C*14, DRB1*04, DRB1*07* e *DRB1*08* tinham frequências diminuídas, não sendo igualmente os valores de *p*, quando corrigidos para as múltiplas comparações, estatisticamente significativos. Em relação à apresentação clínica, os alelos *DRB1*03* (RR=2,39) e *DQB1*02* (RR=2,05) associaram-se de forma significativa com os doentes com síndrome de *Löfgren*, mantendo-se os valores de p estatisticamente significativos depois de corrigidos para as múltiplas comparações (pc=0,01 e pc=0,02 respetivamente).

As frequências do alelo *DRB1*04* mostraram-se aumentadas nos doentes que evoluiram para a cronicidade quando comparadas com a dos que apresentaram regressão (valores estatisticamente não significativos, após correcções de p). Já os alelos *A*01, B*18* e *DRB*03* tinham as suas frequências diminuídas nos doentes que evoluem para cronicidade, não sendo igualmente os valores de *p*, quando corrigidos, estatisticamente significativos.

	Sarcoidoise		Controlos					
Alelo	**n**	**%**	**n**	**%**	**p**	**pc**	**OR**	**95% CI**
B*44	17	8,2%	185	14,2%	0,02	ns	0,54	]0,31;0,92[
B*51	13	6,3%	138	10,6%	0,05	ns	0,56	]0,3;1,04[
C*14	1	0,5%	42	3,2%	0,03	ns	0,14	]0,0;0,86[
DRB1*04	17	8,2%	180	13,8%	0,02	ns	0,55	]0,32;0,95[
DRB1*07	22	10,6%	225	17,3%	0,01	ns	0,57	]0,35;0,92[
DRB1*08	4	1,9%	69	5,3%	0,03	ns	0,35	]0,09;0,95[
B*08	22	10,60%	79	6,10%	0,02	ns	1,83	]1,08;3,08[
DRB1*03	29	13,90%	119	9,20%	0,03	ns	1,61	]1,02;2,53[
DRB1*12	9	4,30%	22	1,70%	0,03(f)	ns	2,63	]1,1;6,09[

(f) valor para o teste exacto de Fisher; **ns** valores estatisticamente não significativos.

Tabela 3 Comparação das frequências alélicas entre os doentes com sarcoidose e a população controlo. Na amostra de doentes estudada, foram observadas relações entre haplótipos quer da classe 1 quer da classe 2 do HLA e a suscetibilidade da doença, que no entanto não se revelaram significativas na correcção para as múltiplas comparações (pc) (Adaptado da referência 152).

No processo fisiopatológico da sarcoidose, as citocinas são fundamentais para a comunicação celular, sendo consequentemente elementos determinantes na modulação da resposta imune.[153-156] Vários estudos têm permitido estabelecer uma conexão entre determinados genótipos das citocinas participantes na resposta imune da sarcoidose e os níveis das mesmas.[152, 156-160] Mutações pontuais ou substituições de nucleótidos na região reguladora dos genes das citocinas afetam a produção das mesmas, podendo ser determinantes na suscetibilidade genética à doença e/ou na dinâmica da resposta imunológica. No mesmo grupo de doentes previamente tipados para o HLA, foram estudados polimorfismos genéticos de citocinas pró e anti-inflamatórias, avaliando a sua influência na suscetibilidade, apresentação clínica e evolução da doença, sendo 83 doentes tipados para o TNF-α e 110 para o TGFβ1, IL-10, IL-6 e IFN-γ. Relativamente às citocinas pró-inflamatórias, os resultados revelaram que o genótipo IFN-γ T/T (*high*) se associou significativamente com a suscetibilidade (p=0,03) e com a presença de uma síndrome ventilatória obstrutiva (p=0,05), para além de uma frequência aumentada do genotipo A/A (*high*) do TNF-α (p=0,04) nos doentes com síndrome de *Löfgren*, não tendo sido encontradas associações significativas com os polimorfismos da IL-6. Nas citocinas anti-inflamatórias, o genótipo IL-10 ATA/ATA (*low*) relacionou-se significativamente com o risco (p=0,04; O.R.=2,12) enquanto o IL-10 GCC/ACC (int) foi protector (p=0,007; O.R.=0,43). O genótipo IL-10 GCC/GCC

(*high*) associou-se significativamente à síndrome de *Löfgren* (p=0,01), enquanto o genótipo IL-10 ATA/ATA (*low*) associou-se significativamente com o envolvimento extra-torácico (p=0,04), cutâneo (p=0,05), elevação da ECA (p=0,03) e com a evolução para a cronicidade (p=0,05). Relativamente ao TGF-β, não se verificou qualquer relação com a suscetibilidade à doença. No entanto, o genótipo TGF-β C/C C/C (*low*) associou-se ao eritema nodoso (p=0.04) e o genótipo T/T G/G (*high*) à síndroma ventilatória restritiva (p=0.05) e à evolução para a cronicidade (p=0.04). Estes resultados parecem confirmar que a suscetibilidade e evolução clínica na sarcoidose são influenciadas por genes que codificam citocinas pró e anti-inflamatórias, podendo ser determinantes nessa relação alguns polimorfismos nas suas regiões promotoras.

Devido ao elevado grau de desequilíbrio de ligação (LD) dentro do MHC não é claro serem os genes HLA a determinar diretamente a suscetibilidade ou pelo contrário, as associações encontradas serem devidas a outros genes em ligação com esta região.[147, 148, 161, 162] Um exemplo é o caso do gene *butyrophilin-like 2* (BTNL2), um membro da superfamília das imunoglobulinas localizado na junção das regiões de HLA de classe II e classe III.[161, 162] *Valentonyte et al.*, descreveram pela primeira vez uma nova associação com o polimorfismo *rs2076530*, resultante de uma mutação (G>A), que resulta numa proteína truncada que afeta a sua localização e função na membrana celular. O *BTNL2* parece ter um papel na modulação dos recetores envolvidos na co-estimulação das respostas das células T, com base na sua homologia de aminoácidos para o CD80/CD86, família de proteínas co-estimulatórias.[163-165] Como consequência, a disfunção da proteína resultante, poderia impedir a regulação das células T e a resposta aos antigénios. Na sequência desta publicação inicial, outros estudos de associação entre polimorfismos *BTNL2* e sarcoidose tem evidenciado resultados conflituantes em populações de diferentes áreas geográficas ou diferentes fenótipos da doença.[166, 167] Efetivamente, foi descrita a ligação do SNP *rs2076530A* do *BTNL2* com a suscetibilidade à sarcoidose em três estudos relacionados com populações de diferentes origens.[166-168] No entanto, num dos estudos referidos, efetuado numa população norte-americana, em que foram incluídos indivíduos de raça branca conjuntamente com outros de raça negra, esta associação só se verificava nos primeiros, enquanto noutro estudo, numa população holandesa, após terem sido retirados os doentes com síndrome de *Löfgren*, esta mesma associação desaparecia, observando-se sim uma associação com outro polimorfismo do *BTNL2*, o *rs2076530C*.[166, 167] Por outro lado, mantém-se por esclarecer se as associações do *BTNL2* são independentes de alelos HLA-*DRB1*.[167, 169, 170] Apesar de se considerarem os polimorfismos do gene *BTNL2* como os mais importantes dos reportados até ao momento no que concerne à suscetibilidade da doença, continuam por definir quais os fenótipos associados, para além da sua eventual influência na evolução da doença.

Mais recentemente, *Hofmann et al.*, publicou uma investigação exaustiva no genoma, incluindo mutações genéticas desconhecidas associadas com a sarcoidose avaliando mais de 440.000 SNPs numa população de 499 doentes e 500 controlos de origem germânica.[171] O estudo evidenciou o SNP *rs1049550* C/T do gene de anexina A11 no cromossoma 10q22.3 como a associação mais significativa com a suscetibilidade da doença, tendo este resultado sido replicado em populações de origem checa e norte-americana.[171-173] A Anexina A11 é um membro da família anexina de proteínas de ligação de fosfolípidos dependentes de cálcio, tendo esta variante funcional denominada *ANXA11* R230C, sido apontada como um marcador de protecção e modificador da doença na sarcoidose.[171-174] A mutação está localizada no exão 6 e resulta numa substituição de uma citosina por uma timidina (C/T), que altera o codão de uma arginina básica para uma cisteína polar no resíduo 230 (R230).[175] Embora as consequências funcionais do SNP *rs1049550* C/T não tenham sido totalmente elucidadas, uma depleção ou disfunção de anexina A11 pode afectar a via de apoptose, induzindo um desequilíbrio entre a apoptose e a sobrevivência de células inflamatórias ativadas.[164, 175-178]

A evolução clínica na sarcoidose é altamente variável, englobando casos de resolução espontânea até outros de deterioração clínica e surgimento de fibrose pulmonar, apesar de uma intervenção terapêutica adequada.[49, 54, 76, 77] Estas diferenças poderão, em certa medida, estar relacionadas com uma interligação de vários genes que influenciam a imunopatogénese e o curso clínico da sarcoidose.[147, 148, 151] Para além da avaliação de cada um dos polimorfismos genéticos que mostram associação à sarcoidose, seria também útil a investigação de uma possível interação entre aqueles que mostraram maior associação com a doença - HLA, *BTNL2*, *ANXA11* - com o objetivo de evidenciar um perfil genético em associação preferencial com determinado tipo de evolução, podendo assim funcionar clinicamente como um marcador de prognóstico.

Como já descrito anteriormente, os diferentes polimorfismos genéticos relacionados com a doença influenciam a expressão de determinadas proteínas envolvidas nos mecanismos patogénicos da sarcoidose podendo, concomitantemente, vir a fornecer marcadores quer de diagnóstico, quer de associação a certos fenótipos ou a determinada evolução clínica. É exemplo a descrição da predisposição para a inflamação crónica por parte de genes que codificam as moléculas de adesão, designadamente o gene ITGAE, que codifica a unidade αE da integrina CD103 (αEβ7) e que nos seus polimorfismos - 1088 A/G e 86141 A/G apresenta uma associação à sarcoidose.[179] A integrina CD103 (αEβ7) correspondente a uma molécula de adesão e migração seletiva que se liga à E-caderina, molécula esta vital para a adesão e retenção de células epiteliais, é expressa também em linfócitos T intra-epiteliais na sua fase terminal de diferenciação.[180-182] Para além da associação à sarcoidose, os referidos polimorfismos do ITGAE encontram-se associados às formas mais graves da sarcoidose pulmonar, nomeadamente ao estadio IV, onde se verificam igualmente níveis

aumentados de linfócitos CD4+CD103+ no BAL.[179, 183, 184] Efetivamente as células CD4+CD103+ encontram-se fundamentalmente no compartimento das células T de memória e têm uma capacidade reguladora, suprimindo a proliferação celular.[185] Adicionalmente, quando ativada, esta subpopulação celular pode divergir para um padrão de citocinas Th2, com particular enfase na produção de IL-13, que tem propriedades pró-fibróticas e medeia a indução da fibrose dependente da TGF-β1.[185, 186] No entanto, os níveis de CD4+CD103+ no BAL dos doentes com sarcoidose têm sido descritos como caracteristicamente diminuídos, possivelmente em relação com o recrutamento da periferia dos linfócitos que participam na inflamação a nível pulmonar, ao invés de outras doenças inflamatórias de predomínio linfocitário, que se podem acompanhar preferencialmente de uma expansão local dos linfócitos presentes na mucosa do trato respiratório (i.e. CD103+), o que poderá ter implicações também no diagnóstico diferencial, nomeadamente nas doenças pulmonares granulomatosas.[184, 187-189] Estas observações podem ser importantes para o diagnóstico de sarcoidose e da sua evolução, nomeadamente através da análise no BAL da expressão de células CD4+CD103+.

Como referido anteriormente, entre as ILD, aquelas que se caracterizam por uma reacção inflamatória granulomatosa são especialmente relevantes pela sua prevalência.[25] A sarcoidose e a pneumonite de hipersensibilidade (HP), sendo as mais comuns deste grupo, diferem no entanto em muitos outros aspetos.[1, 4, 25] A sarcoidose é uma doença sistémica, com apresentações clínicas heterogéneas, caracterizadas pela acumulação de linfócitos auxiliares CD4+Th1 nos orgãos afetados.[47, 49, 52] A PH, restrita apenas ao pulmão, é igualmente caracterizada por uma inflamação linfocítica nas vias aéreas periféricas e tecido intersticial circundante, com formação de granulomas.[81, 89] Estes parecem ser o resultado de uma reação de hipersensibilidade retardada clássica, mediada por linfócitos T, à inalação repetida de determinado antigénio.[81, 89] No entanto, a linfocitose no BAL é de predomínio CD8+, resultando habitualmente numa relação CD4+/CD8+ diminuída.[97] Também na HP, existem variações assinaláveis deste valor, tendo sido demonstrados valores mais elevados na apresentação crónica e em alguns antigénios associados (ex. exposição a proteínas aviárias), motivo pelo qual uma relação CD4+/CD8+ elevada não excluí o diagnóstico de HP.[83, 91, 123] Pouco se sabe acerca da expressão de CD103 na HP, mas tratando-se de uma doença por hipersensibilidade imunológica associada à inalação de antigénios, é plausível que a alveolite linfocítica presente na HP resulte de uma expansão mucosa / intraepitelial local em contraste com um recrutamento da periferia, como acontece na sarcoidose.

A HP é no entanto uma doença com uma heterogeneidade significativa, quer na sua forma de expressão, quer no tipo de evolução.[89, 91] Efetivamente, paralelamente a formas autolimitadas, que podem inclusive regredir totalmente após cessação da exposição antigénica, eventualmente acompanhada por corticoterapia, existem formas de HP crónicas com apresentação clínica e imagiológica semelhantes a algumas IIP, como a IPF

ou a NSIP fibrosante.[25, 91] Para além desta forma de apresentação, em alguns casos, verifica-se uma evolução progressivamente desfavorável, com alastramento da fibrose e insuficiência respiratória, verificando-se uma sobrevida limitada, semelhante à que se observa habitualmente na IPF.[25, 91] Pela semelhança de apresentação e evolução, a HP crónica, nomeadamente a que se associa a um padrão de UIP na HRCT poderá apresentar mecanismos fisiopatológicos semelhantes, embora num contexto diferente de uma doença inflamatória que evolui para a fibrose em contraste com um processo eminentemente pró-fibrótico que caracteriza a IPF.[190] Embora a sarcoidose apresente formas de fibrose pulmonar, esta habitualmente é caracterizada por um prognóstico mais favorável e por características clínicas e imagiológicas distintas da HP crónica e da IPF, podendo, no entanto, raramente haver formas que se aproximam daquelas duas entidades.[77, 191] Por sua vez, a IPF é uma doença pulmonar crónica fibrosante caracterizada por uma deterioração funcional progressiva e uma sobrevida média de 2 a 3 anos a partir da altura do diagnóstico.[11, 12] Associa-se com um padrão radiológico e histológico de UIP e é a forma mais comum de pneumonia intersticial idiopática.[11, 12] Apesar da sua etiopatogenia ser em grande parte desconhecida, a hipótese mais considerada atesta ser a IPF resultante de um processo de reparação alterado em resposta a uma agressão epitelial alveolar recorrente (cuja natureza é desconhecida), tendo como consequência um processo fibroproliferativo persistente e progressivo.[12, 13, 192] É possível que estas três patologias diferentes - IPF e as formas fibrosantes de HP e sarcoidose - apresentem alguns elos comuns na desregulação da reparação/remodelação tecidular, na sequência da agressão epitelial alveolar.

As metaloproteinases da matriz extracelular (MMPs) pertencentes ao grupo das endoproteases dependentes do zinco, apresentam propriedades pró-fibróticas, ao participarem na remodelação da matriz extracelular, na cicatrização ou na angiogénese.[193, 194] Efetivamente, foi descrito que ratos *Knockout* MMP-7 apresentaram protecção para a fibrose induzida pela bleomicina.[195] Embora tenham sido descritas diferenças significativas da expressão de MMP-1 e MMP-7 na IPF em comparação com outras ILDs, nomeadamente com a sarcoidose e HP (maioritariamente em formas subagudas), uma comparação direta com as entidades que constituem uma maior dificuldade no diagnóstico diferencial, isto é as UIP secundárias, nomeadamente associadas à HP e a NSIP fibrosante, mantém-se por realizar.[196] Efetivamente existe uma sobreposição significativa relativamente ao padrão UIP, podendo este estar no contexto da IPF, mas também da HP, conectivites ou ainda resultante da toxicidade induzida por algumas drogas como a amiodarona, situação que se verifica igualmente com o padrão NSIP.[10, 11] O facto de apresentarem alterações imagiológicas e patológicas semelhantes, poderá indicar que poderão ter um mecanismo fisiopatológico igualmente semelhante, independentemente de serem integrados em entidades nosológicas diferentes, podendo eventualmente necessitar de uma abordagem direcionada para essa expressão particular, em contraste da interpretação clínica habitual que tem em conta principalmente a designação tradicional da doença.

Objetivos

Tendo em conta as já estabelecidas influências imunogenéticas e imunopatogénicas na suscetibilidade, expressão clínica e evolução das doenças do interstício pulmonar, foram objetivos desta dissertação:

1. Avaliar a associação do alelo *rs2076530* G/A do *BTNL2*, um membro da superfamília das imunoglobulinas localizado na junção das regiões da classe II e classe III do HLA, com a suscetibilidade à sarcoidose numa população do Norte de Portugal, antes e após a estratificação dos seus diferentes fenótipos de apresentação e tendo em conta a distinta evolução clínica da doença.

2. Avaliar se o polimorfismo genético *rs1049550* C/T da anexina A11 (variante R230C), uma das associações genéticas mais relevantes na suscetibilidade à sarcoidose em estudos populacionais, é igualmente um marcador de suscetibilidade numa população do Norte de Portugal.

3. Dado que a anexina A11 pode afetar as vias de apoptose e a sobrevivência de células inflamatórias ativadas, pretende-se avaliar igualmente uma associação da sua variante R230C com os diferentes fenótipos de apresentação e evolução clínica da sarcoidose.

4. Investigar uma possível interação entre os determinantes imunogenéticos com maior associação à sarcoidose na população caucasiana - HLA, *BTNL2*, *ANXA11* - com o objetivo de evidenciar perfis genéticos potencialmente associados às diferentes formas de evolução desta doença.

5. Estudar a expressão da integrina CD103 (**αEβ**7), uma molécula de adesão ligando da E-caderina e expressa em linfócitos T intra-epiteliais na sua fase terminal de diferenciação, nas subpopulações linfocitárias de doenças do interstício pulmonar que se caracterizam por recrutamento linfocitário ao pulmão e eventual evolução fibrosante.

6. Avaliar os níveis séricos de MMP-1 e MMP-7, duas metaloproteinases que participam na remodelação da matriz extracelular e angiogénese, nas doenças do interstício pulmonar, designadamente na fibrose pulmonar idiopática e nas formas fibrosantes de pneumonite de hipersensibilidade e sarcoidose, patologias com possíveis elos comuns na desregulação da reparação tecidular.

Materiais e Métodos

PARTICIPANTES

DOENTES

Estudo I

Foram incluídos 151 doentes caucasianos e sem relação familiar entre eles, originários da região norte de Portugal. A média de idades era de 38±4,2 anos e 57% eram mulheres. Todos os doentes tinham envolvimento torácico pela sarcoidose, diagnosticado através da avaliação do quadro clínico, para além da análise funcional respiratória e imagiológica. Em 66% dos doentes foi obtida histologia compatível com o diagnóstico de sarcoidose.

Estudo II

Foram incluídos 208 doentes caucasianos e sem relação familiar entre eles, originários da região norte de Portugal. A média de idades era de 38 ± 12anos e 59% eram mulheres. Todos os doentes tinham envolvimento torácico pela sarcoidose, diagnosticado através da avaliação do quadro clínico, para além da análise funcional respiratória e imagiológica. Em 69% dos doentes foi obtida histologia compatível com o diagnóstico de sarcoidose.

Estudo III

Foram incluídos 138 doentes caucasianos e sem relação familiar entre eles, originários da região norte de Portugal. A média de idades era de 37,2±12,1 anos e 57% eram mulheres. Em 99% dos doentes foi observado envolvimento torácico pela sarcoidose, diagnosticado através da avaliação do quadro clínico, para além da análise funcional respiratória e imagiológica. Em 66% dos doentes foi obtida histologia compatível com o diagnóstico de sarcoidose.

Estudos IV e V

Foram incluídos 86 doentes com ILD (idade média de 42,6±16,6 anos, sendo 61% mulheres), submetidos a BAL na abordagem diagnóstica. Com o objectivo de comparação, os doentes foram divididos entre doentes com sarcoidose (n-41) e outras ILD (n-45). Foram incluídos 22 doentes com o diagnóstico de HP, efetuado de acordo com os critérios de *Schuyler et al.*[197] O diagnóstico de IIP foi efetuado de acordo com as normas *ATS/ERS International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias*: IPF-4, NSIP-3, OP-1.[10] O envolvimento pulmonar

por conetivites assentou nos critérios propostos pela *European League Against Rheumatism* (EULAR) e na evidência de critérios imagiológicos na HRCT torácica de pneumonia interstitical em doentes previamente diagnosticados com doença auto-imune: lupus eritematoso sistémico-3, esclerose sistémica-2, artrite reumatóide-3.[7] Foram igualmente incluídos 4 doentes com toxicidade pulmonar induzida por drogas (3 secundária a sirolimus e 1 a capacitabina) e 3 doentes com silicose, cujo diagnóstico foi baseado em exposição compatível, adicionado a alterações imagiológicas e do BAL caraterísticas.

Estudo VI

Foram incluídos 139 doentes com ILD, divididos em 3 grupos: IPF (n =47), UIP não IPF (n = 36), e NSIP fibrótica (n = 43). O grupo de doentes com UIP não IPF englobava 21 doentes com HP (12 com doença dos criadores de aves, 6 com suberose e 3 com etiologia desconhecida), 13 com ILD no contexto de conetivite (7 com esclerose sistémica, 5 com artrite reumatóide e 1 com conetivite indiferenciada) e 2 doentes com toxicidade pulmonar secundária à amiodarona. O grupo com NSIP fibrótica englobava 14 doentes com NSIP idiopática e 29 com NSIP no contexto de conetivite (13 com esclerose sistémica, 8 com artrite reumatóide, 3 com síndrome de *Sjogren*, 2 com conectivite mista, 2 com dermatopolimiosite e 1 com lupus eritematoso sistémico). Foram igualmente incluídos 13 doentes com sarcoidose em estadio IV e um grupo controlo de 20 indivíduos saudáveis (idade média 70,3±6,4 anos, 55% homens).

Os doentes com IPF foram diagnosticados de acordo com as normas ATS/ERS publicadas em 2011.[11] Os doentes com conectivites foram diagnosticados de acordo com as recomendações da EULAR, tendo sido o envolvimento torácico diagnosticado pelas alterações a nível da HRCT torácica e do BAL.[7] Os doentes com HP foram diagnosticados de acordo com os diagnósticos propostos por *Lacasse et al*.[90]

Estudos I, II, III, IV, V, VI: Critérios de diagnóstico

Para o diagnóstico de sarcoidose, todos os doentes sem confirmação histológica cumpriam os critérios de diagnóstico estabelecido pelas normas de actuação ERS/ATS/WASOG, nomeadamente um quadro clínico e alterações radiológicas compatíveis, BAL com linfocitose associada a uma relação CD4/CD8> 4 e um período de observação de pelo menos 2 anos de forma a outras condições clínicas serem excluídas com maior segurança.[45] A síndrome de *Löfgren* foi definida pela combinação da presença de adenopatias hilares bilaterais simétricas, febre, artralgias dos membros inferiores e eritema nodoso.[45] O envolvimento torácico pela sarcoidose foi classificado segundo os critérios de *Scadding*, na altura do diagnóstico (estadio 0: sem sinais de alterações torácicas; estadio I: adenopatias mediastínicas e sem envolvimento pulmonar; estadio II: adenopatias mediastínicas e envolvimento pulmonar; estadio III: apenas envolvimento pulmonar, e estadio IV: fibrose pulmonar).[57] Relativamente à evolução, a resolução da doença foi considerada quando se observou ausência de sintomas e de alterações funcionais respiratórias ou radiológicas

durante os primeiros 2 anos após o diagnóstico. Ao contrário, foi considerado doença crónica após 2 anos de manutenção de qualquer das alterações referidas. Todos os doentes incluídos (estudos I-VI) foram diagnosticados após avaliação multidisciplinar que incluiu 3 pneumologistas, 2 radiologistas e 2 patologistas, experientes na observação de doentes com ILD.

GRUPO CONTROLO

Estudo I
O estudo englobou um grupo controlo de 150 indivíduos caucasianos, dadores voluntários de medula óssea, escolhidos aleatoriamente e provenientes da mesma região geográfica dos doentes. Tinham uma média de idades de 51,9±14,5 anos e 51% eram mulheres. Em todos os indivíduos foi despistada doença respiratória, não apresentando nenhum deles sintomas respiratórios ou alterações sugestivas de doença nos exames séricos ou em radiografia torácica.

Estudo II
O estudo englobou um grupo controlo de 197 indivíduos caucasianos, dadores voluntários de medula óssea, escolhidos aleatoriamente e provenientes da mesma região geográfica dos doentes. Tinham uma média de idades de 31,8±7,0 anos e 62% eram mulheres. Em todos os indivíduos foi despistada doença respiratória, não apresentando nenhum deles sintomas respiratórios ou alterações sugestivas de doença nos exames séricos ou em radiografia torácica.

MÉTODOS

ESTUDOS GENÉTICOS (Estudos I, II e III)

A extracção de ADN dos doentes e controlos foi feita a partir do sangue periférico colhido em tubos com ACD (*acid citrate dextrose*) com *QIAsymphony* ou *QIAmp Kit* (Qiagen, Venlo, Netherlands).

Foi feita a pesquisa de transição G/A na posição c.1078A>G com o *rs2076530* (NCBI dbSNP. http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2076530) do gene *BTNL2* utilizando o kit de genotipagem *TaqMan SNPs genotyping assay* (*Applied Biosystems*, Foster City, CA, USA) com sondas marcadas respetivamente com VIC e FAM.

A reacção de PCR foi efetuada num volume total de reacção de 12,5Ql adicionando 20ng de ADN de acordo com as instruções do fabricante. O protocolo de amplificação inclui a: desnaturação a 95ºC durante 10 min, seguido de 40 ciclos de desnaturação a 92ºC em 15 seg e anelamento e extensão a 60ºC por 1min. Após o PCR, a genotipagem foi determinada por fluorescência específica alélica em ABI PRIM 7000 *Sequence Detection*

Systems (*Applied Biosystems*, Foster City, CA, USA) utilizando o *software* SDS 1.1 para descriminação alélica.

As amostras de ADN foram também genotipadas para *DQB1* HLA-A, B, Cw, *DRB1* e *DQB1* por PCR - *Sequence Specific Oligonucleotides* (PCRSSO) com *Luminex technology* (*Tepnel Lifecodes Kits*, Stamford). A amplificação do ADN foi efectuada num volume total de 25QL com 7.5 QL de *MasterMix* especifica para cada *locus* dos genes estudados, 1.25U de *GoTaqHot Start polymerase* (Promega, Madison WI) e 50ng ADN genómico. A reação de PCR inclui: 5min de 95º, 95 ºC de 30s, 60 ºC- 45s, 72 ºC - 45s, acrescentando 32 ciclos de 95 ºC 30s, 63 ºC 45s, 72 ºC 45s e no final 72 ºC de 15 min. Os 2.5 QL de produto de amplificação obtidos foram misturados com 7.5 QL sondas de *Luminex* previamente aquecidos a 58ºC, e a hibridização foi efetuada segundo o protocolo, em resumo: 97ºC: 5min, 47ºC: 30min e 56ºC: 10min. Foram usadas 100 QL de diluente com 0.4 QL de *Streptavidin-PE* (*Tepnel Lifecodes*, Stamford) de 56ºC para a fase terminal de hibridização. O *Quicktype LifeMatch software v2.5* (*Tepnel Lifecodes*) foi utilizado para analisar o HLA.

LAVADO BRONCOALVEOLAR E CITOMETRIA DE FLUXO (Estudos IV e V)

O BAL foi realizado de acordo com as recomendações da ERS, recorrendo ao uso de um broncofibroscópio. Quatro seringas de 50 ml de solução salina isotónica estéril (37 ºC) são instiladas no lobo médio e suavemente aspiradas com uma seringa (após cada instilação). O volume recuperado de BAL foi misturado (rejeitando a primeira seringa), após filtração por uma gaze e o número total de células (câmara de *Neubauer*) determinado. A contagem celular diferencial foi obtida por meio da contagem de 500 células em preparações de lâminas coradas com *Wright-Giemsa*. Para a análise fenotípica, as células foram centrifugadas a 250 g durante 10 min, lavadas duas vezes, ressuspensas, e marcadas com a seguinte combinação de anticorpos monoclonais: anti-CD103-FITC, anti-CD8-Pe, anti-CD45-PercP-Cy.5 e anti-CD4-APC (*Becton Dickinson Immunocytometry Systems*, BDIS, San José, CA, USA). As expressões de CD3, CD19, e CD56 foram igualmente estudadas em paralelo na amostra. As células foram adquiridas em citómetro de fluxo (FACScan) usando o *Software CellQuest-Pro* (*Beckton Dickinson* e *BD Biosciences*, San Jose', EUA). O *gate* dos linfócitos foi feito com base em dispersão frontal (FSC) *versus* dispersão lateral (SSC). O *gate* adicional foi baseado em SSC *versus* expressão face aos marcadores CD45, CD4 e CD8. No BAL os linfócitos T foram analisados quanto à expressão de CD4 +, CD8 +, CD103 + CD4 + e CD8 + CD103+.

QUANTIFICAÇÕES SEROLÓGICAS (Estudo VI)

As amostras de sangue foram obtidas por punção venosa para tubos com soro de gel *Terumo Venosafe*. O soro foi separado após 30 min de colheita seguido de centrifugação 10 min a 400x g. As amostras de soro foram guardadas a -80ºC até à sua utilização. MMP-1 e MMP-7 foram doseadas

por multiplex (R&D Systems, Inc.) de acordo com as instruções do fabricante. Em resumo, o ensaio é baseado na tecnologia de MAP *multiplex Fluorokine* (*Luminex Corporation*), que combina o princípio de um imunoensaio em sanduíche com a tecnologia baseada na fluorescência emitida por esferas. Os padrões e as amostras de soro foram diluídas (1:10) com diluente e adicionadas à microplaca pré-humedecida. Esferas contendo anticorpos fluorescentes anti-MMP-1 e anti-MMP-7 foram adicionadas aos poços da microplaca e esta incubada num agitador durante 2h à temperatura ambiente. Após a lavagem, anticorpos biotilanizados são adicionados a cada poço e incubam durante 1h à temperatura ambiente, lavados e incubados por mais 30 min. com o conjugado *streptavidin-phycoerythrin*. Após a lavagem, as esferas são resuspensas em tampão de lavagem e analisadas no aparelho Luminex 200. A intensidade média de fluorescência foi obtida por integração no sistema do Luminex 100 versão 2.3. A geração da curva padrão com base nos padrões com diferentes concentrações de MMP permitiu o doseamento individual de cada metaloproteinase. O limite de detecção para a MMP-1 e MMP-7 foi 4,4 e 16,9 pg/ml, respetivamente.

ANÁLISE ESTATÍSTICA

Estudo I, II, III

A análise descritiva foi resumida em tabelas para todas as variáveis analisadas. As variáveis contínuas são sumariadas com a média, mediana, desvio padrão e intervalo interquartil. As frequências de alelos e genótipos foram obtidas através de contagem direta: frequência alélica (%)=(n/2N)x100, onde n indica a soma de um alelo em particular e N o numero de indivíduos. Os desvios do equilíbrio de *Hardy–Weinberg* foram testados com o *software* Arlequin v3 usando o método descrito por *Guo and Thompson*.[204]

As comparações das frequências alélicas, genotipicas e haplotipicas entre os diferentes grupos de doentes, foi feita através do teste do x2 (ou teste exacto de *Fisher* quando apropriado) na análise univariada e com recurso à regressão logística na análise multivariada. Os Riscos Relativos ou *Odds Ratios* (OR) e respetivos intervalos de confiança a 95% (95% CIs) foram calculados enquanto medidas de associação.

As análises foram efetuadas com os *softwares* Statcalc (EpiInfo 2002, *Centers for Disease Control and Prevention*, Atlanta, GA, USA) e SPSS v19. Foi utilizado um nível de significância estatística de 5% para a análise dos valores *p*. Estes valores *p* foram corrigidos (pc) para as múltiplas comparações através do método de *Bonferroni*.

Devido ao Desequilíbrio de Ligação (LD) entre os alelos de HLA e *BTNL2*, foi feita a análise de regressão logística para determinar o potencial de confundimento e efeito modificador dos alelos do *BTNL2* em relação aos alelos de risco da classe II do HLA. Na análise haplotípica, foram investigadas as medidas dos LD emparelhados e construídos dos haplótipos HLA-*DRB1*-DQB1*-BTNL2* através do algorítmico da maximização do valor esperado

(EM) implementado no *software* Arlequin.[205] O LD entre os pares de loci seleccionados foi testado usando o teste de verosimilhança para a significância da associação entre os pares de loci, onde a verosimilhança da amostra avaliada sob a hipótese de não associação entre loci (*linkage equilibrium*) é comparada com a verosimilhança da amostra quando a associação é permitida. Foi testado o LD entre dois alelos de dois loci diferentes, construindo tabelas de contingência 2x2 com os valores individuais (observados) classificados por nível em dois atributos diferentes, isto é, dois alelos de dois locis diferentes.

Alelos HLA, *BTNL2*, e *ANXA* foram incluídos nos modelos de regressão como preditores independentes para a evolução na sarcoidose. Termos de interacção foram incorporados nos modelos de regressão logística para avaliar possíveis interações multiplicativas entre os alelos.

Estudos IV, V, VI

Os resultados foram descritos como média e desvio padrão ou como mediana e intervalo interquartil (IQR) para variáveis quantitativas e como contagens e proporções. Foi utilizado o teste *t-Student* e o teste *Mann-Whitney*, a ANOVA ou o teste de *Kurskal-Wallis* para comparar as médias e medianas das variáveis quantitativas e de acordo com a normalidade das distribuições.

As associações entre a concentração de MMP-1 e MMP-7 com cada diagnóstico foram estimadas através de modelos de regressão logística, ajustados para a idade, sexo e história tabágica (nunca *vs* ex ou fumador).

Sensibilidade e especificidade foram calculadas para uma série de pontos de corte para as várias formas de expressão de CD103 nos linfócitos $CD4^+$ e $CD8^+$ do BAL. A área abaixo (AUC) da curva ROC (*receiver operating characteristic*), cujos eixos são a sensibilidade *vs* 1-especificidade, foi utilizada para descrever a performance de diagnóstico da MMP-1 e MMP-7. Valores preditivos positivos e negativos (PPV e NPV) foram também estimados.

As curvas ROC foram igualmente utilizadas para determinar a AUC para MMP-1, MMP-7 e MMP-1/MMP-7 combinado no que respeita ao diagnóstico de IPF *vs* qualquer outro diagnóstico (excluindo controlos). Sensibilidade, especificidade, valores preditivos positivos e negativos (VPP e VPN) respetivamente) foram calculados para os *cut-offs* de MMP-1 e MMP-7 e o índex de *Youden* (J = max [*sensitivity* + *specificity*-1]) foi usado para estabelecer o melhor *cut-off* para o diagnóstico de IPF.

A análise estatística foi realizada recorrendo ao programa SPSS versão 18 do Windows (SPSS; Chicago, IL, USA). A significância estatística foi atribuída para valores p abaixo de 0,05 para todos os testes realizados.

ÉTICA

Todos os indivíduos incluídos nos vários estudos apresentados assinaram um consentimento informado. Os estudos foram submetidos e aprovados pela comissão de ética do Centro Hospitalar de São João.

Publicações

BTNL2 gene polymorphism associations with susceptibility and phenotype expression in sarcoidosis

António Morais, Bruno Lima, Maria José Peixoto, Helena Alves, Agostinho Marques, Luís Delgado

Respiratory Medicine (2012) 106, 1771-1777

Respiratory Medicine (2012) **106**, 1771–1777

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/rmed

BTNL2 gene polymorphism associations with susceptibility and phenotype expression in sarcoidosis

António Morais [a,*], Bruno Lima [b], Maria José Peixoto [b], Helena Alves [b], Agostinho Marques [a], Luís Delgado [c,d]

[a] *Pneumology Department, Centro Hospitalar São João, Faculdade de Medicina, Universidade do Porto, 4200-319 Porto, Portugal*
[b] *North Histocompatibility Centre, Portugal*
[c] *Immunology Department, Faculdade de Medicina, Universidade do Porto, 4200-319 Porto, Portugal*
[d] *Centre for Research in Health Technologies and Information Systems (CINTESIS), University of Porto, Portugal*

Received 23 March 2012; accepted 14 August 2012
Available online 25 September 2012

KEYWORDS
Sarcoidosis;
Genetics;
BTNL2;
HLA;
Disease evolution

Summary
A functional polymorphism within butyrophilin-like 2 (*BTNL2*) gene has been described as a potential risk factor for sarcoidosis. The association between chronicity and the *rs2076530* SNP *A* allele has also been reported.

This study evaluates the *BTNL2 rs2076530* G/A allele associations with sarcoidosis susceptibility and disease evolution in a Portuguese cohort of patients.

A case-control study of 151 patients and 150 controls was performed. Allele frequencies were compared with Chi-square test in a univariate analysis and with logistic regression in a multivariate analysis.

BTNL2 rs206530 A allele frequencies were significantly higher in sarcoidosis with no linkage disequilibrium with HLA-*DRB1* alleles, except in the subgroup of patients with Löfgren syndrome where the determinant allele was HLA-*DRB1*03*. The *A* allele was also increased in those with isolated thoracic disease, with no differences regarding radiological stages or disease evolution. HLA-*DRB1*03*, besides the association with Löfgren syndrome was significantly related with disease resolution. Our data confirms the association of *BTNL2 rs2076530 A* allele with sarcoidosis susceptibility in a Portuguese population. We found independent genetic risk factors in clinically distinct disease phenotypes: *BTNL2 rs2076530 A* allele in patients without Löfgren syndrome or with isolated thoracic disease, and HLA-*DRB1*03* in Löfgren syndrome or disease resolution.

* Corresponding author. Serviço de Pneumologia, Centro Hospitalar São João, Alameda Prof. Hernani Monteiro, 4200-319, Porto, Portugal. Tel.: +351 919175999.
E-mail address: amorais3@hotmail.com (A. Morais).

0954-6111/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.rmed.2012.08.009

Introduction

Sarcoidosis is a multiorgan granulomatous inflammatory disease of unknown etiology, characterized by activation of macrophages and CD4+ T cells and the formation of non-caseating granulomas, predominantly in the lungs and lymph nodes.[1,2] An underlying interaction between genetic factors and unknown environmental factors is a widely accepted hypothesis for the pathogenesis of sarcoidosis.[3,4] Familiar clustering, the increased risk of relatives to develop sarcoidosis, and different clinical presentations related to distinct ethnic groups, support the role of genetic factors in sarcoidosis susceptibility and heterogeneity.[5,6]

Family-based and case control studies have consistently shown an association between Human Leukocyte Antigen (HLA) haplotypes and sarcoidosis, regarding both disease susceptibility and phenotype expression.[7,8] However due to the high range of linkage disequilibrium (LD) within the major histocompatibility complex (MHC) it remains unclear whether the HLA genes directly determine the susceptibility or the associations are due to other genes in linkage with this region.[9,10] This is the case of butyrophilin-like 2 (*BTNL2*) gene, a member of the immunoglobulin superfamily located at the junction of the HLA class II and class III regions.[11,12]

Valentonyte et al. reported for the first time a novel association with a truncating single nucleotide polymorphism (SNP) *rs2076530* (G → A) of the *BTNL2* gene, independently of the HLA sarcoidosis risk alleles.[11] *BTNL2* seems to have a role in the modulation of costimulatory receptors involved in T-cell responses, on the basis of its amino acid homology to the CD80/CD86 family of costimulatory proteins.[13,14] As a consequence, dysfunction of the BTNL2 protein could impair normal T-cell regulation and response to antigens. Following the initial report, other studies of BTNL2 polymorphisms and sarcoidosis generated conflicting results in populations from different geographic areas or different disease phenotypes.[15,16] On the other hand, whether BTNL2 associations are independent of HLA-*DRB1* alleles remains to be elucidated, in view of contradictory data so far.[11,16–19]

Our purpose was to evaluate *BTNL2 rs2076530* G/A allele as a putative genetic risk for sarcoidosis in a Portuguese population, before and after stratification of clinically distinct disease phenotypes and evolution. Given the variability of the published data from diverse ethnic groups, an evaluation of HLA alleles with LD analyses was also carried out.

Materials and methods

Subjects

For a case-control study with 1:1 ratio, a type 1 error rate of 5%, an 80% of power to detect a modest genotype odds ratio of 1.4 for disease susceptibility, we estimated a sample size with 123 patients and 123 controls. We included a total of 151 unrelated Caucasian patients from the north region of Portugal (mean age 38.0 ± 4.2 years, 57% women) and 150 controls. All patients had thoracic sarcoidosis, as determined by symptoms, radiology and pulmonary function tests, supported by the evidence of non-caseating epitheloid cell granuloma in biopsy specimens in 66%. All subjects without histological confirmation fulfilled the ERS/ATS/WASOG statement criteria,[2] namely compatible clinical and radiographic features, a bronchoalveolar lavage fluid (BALF) lymphocyte CD4/CD8 > 4.0, and a 2 years observation period to exclude other medical conditions. Löfgren's syndrome (LS) was defined as bilateral hilar lymphadenopathy, fever, ankle arthralgia and erythema nodosum.[1–3] Thoracic involvement was classified according with Scadding criteria at the time of diagnosis (stage 0-no thoracic involvement, stage I-adenopathies without lung involvement, stage II-adenopathies and lung involvement, stage III-only lung involvement, IV-lung fibrosis).[20] Disease resolution was considered when a disappearance of symptoms, normalization of chest X-ray and pulmonary function tests occurred within 2 years after diagnosis.[18,19,21] A control group included 150 unrelated and healthy bone marrow donors, randomly recruited from the same geographic region and from the same ethnic background. They had a mean age of 51.9 ± 14.5 years and 51% were women, no history of lung disease, respiratory symptoms or other disease by chest radiography or laboratory blood tests. Written consent was obtained from all subjects, and the study approved by our hospital Ethics Committee.

Genotyping

DNA from patients and controls was obtained from peripheral blood using standard methods. Samples were genotyped for the *BTNL2 G → A* transition of *rs2076530* using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). Minor groove binding probes were labeled with the fluorescent dyes VIC and FAM, respectively. Polymerase chain reaction (PCR) was carried in a total reaction volume of 12.5 μl with TaqMan SNP Genotyping assay 1x, TaqMan Genotyping Master Mix 1x and 20 ng of genomic DNA. The amplification protocol included a denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 92 °C for 15 s and annealing and extension at 60 °C for 1 min. After PCR, the genotype was determined by allelic-specific fluorescence on the ABI PRIM 7000 Sequence Detection Systems and SDS 1.1 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA).

DNA samples were also genotyped for HLA-*A*, *B*, *Cw*, *DRB1* and *DQB1* by PCR-Sequence Specific Oligonucleotides (PCR-SSO) with Luminex technology (Tepnel Lifecodes Kits, Stamford). DNA amplification was carried in a total volume of 25 μl with 7.5 μl of Master Mix specific for each locus, 1.25 U of GoTaq Hot Start Polymerase (Promega, Madison WI) and 50 ng of genomic DNA. PCR was run with the program: 5 min at 95 °C, followed by 8 cycles of 95 °C 30s, 60 °C 45s, 72 °C 45s, with an increment of 32 cycles of 95 °C 30s, 63 °C 45s, 72 °C 45s and a final extension hold of 72 °C for 15 min. After DNA amplification, 2.5 μl of PCR product was mixed with 7.5 μl of pre-warmed Luminex probes (58 °C) and hybridized with the following protocol: 97 °C for 5 min, 47 °C for 30 min and 56 °C for 10 min, prior to Luminex reading, the hybridization stage stopped at 56 °C with 100 μl of a dilution solution combined with 0.4 μl of Streptavidin-PE (Tepnel Lifecodes, Stamford). HLA typing was analyzed with the software Quicktype LifeMatch v2.5 (Tepnel Lifecodes).

Statistical analysis

Allelic and genotypic distributions were statistically analyzed with the χ^2-test (or Fisher exact test when appropriate) in univariate analysis and with logistic regression in multivariate analysis. Relative Risks or Odds ratios (OR) and their 95% confidence intervals (95% CIs) were calculated as association measures. The software used was the Statcalc program (EpiInfo 2002, Centers for Disease Control and Prevention, Atlanta, GA, USA). Regression analyses where performed in SPSS v13 statistical software. *p* Values less than 0.05 were considered statistically significant. *p* Values where corrected (pc) for multiple comparisons using Bonferroni method. HLA data and *BTNL2* haplotypes were analyzed by carriage frequency, while *BTNL2* allele's data by allele frequency unless otherwise stated. Due to the strong LD between many HLA alleles and *BTNL2*, logistic regression analysis was performed to determine the potential for confounding and effect modification of *BTNL2* allelic in relation to HLA class II risk alleles. For haplotype analysis, pairwise LD measures were investigated and HLA-*DRB1*-DQB1*-BTNL2* haplotypes constructed with the expectation-maximization (EM) algorithm implemented in Arlequin software.[22] LD between selected pairs of loci was tested using a likelihood test for the significance of the association between pairs of loci, where the likelihood of the sample evaluated under the hypothesis of no association between loci (linkage equilibrium) is compared to the likelihood of the sample when association is allowed. We tested LD between two alleles from two different loci, constructing a 2×2 contingence table with the individual (observed) values cross-classified by level in the two different attributes, i.e., two alleles from two different loci.

Results

According with Scadding criteria 49 (32.4%) patients were in stage I, 67 (44.3%) in stage II, 14 (9.2%) in stage III and 21 (13.9%) in stage IV. Sixty one (40.3%) patients presented extra-thoracic disease, with skin and liver as the most frequent involved structures, both with 21 (13.9%) patients. Löfgren's syndrome was observed in 29 (19.2%) cases. A consistent clinical course was established in 144 patients (95.4%), 66 (43.7%) presenting disease resolution and 78 (51.7%) chronic forms; among these, 41 (52.6%) had chronic stable and 37 (47.4%) progressive disease.

Disease susceptibility

BTNL2 rs2076530 genotype frequencies and HLA allele frequencies were in Hardy–Weinberg equilibrium across both studied populations, cases and controls. Genotype and allele frequencies were similar to those reported for Caucasians.[23] When allele and genotype frequencies of healthy controls and patients were compared, an association of the *BTNL2 rs2076530* polymorphism with sarcoidosis was noted, with a significant increase of the *rs2076530 A* allele frequency in the sarcoidosis group compared with controls (65.2% vs 55.7%, OR = 1.49, 95% CI = [1.06; 2.10], $p = 0.01$) (Table 1). Association of *BTNL2 rs2076530 AA* genotype with sarcoidosis risk was even stronger (44.4% vs 28.7%, OR = 1.98, 95% CI = [1.20; 3.29], $p = 0.004$).

The HLA alleles associated positively with sarcoidosis on univariate analysis were *A*03*, *B*07*, *DRB1*14*. Otherwise, *A*02*, *A*29*, *B*44*, *Cw*16*, *DRB1*07*, *DRB1*08* were statistically significant as protectors for the disease (Table 1). However, only *Cw*16* remains significantly associated with sarcoidosis after Bonferroni correction for multiple comparisons. Although degree of LD over the HLA genomic region is high and variable,[7,10] LD between *rs2076530 A* allele and *DRB1*14*, an allele previously described in sarcoidosis susceptibility,[24,25] was not statistically significant. To further evaluate the *DRB1*14* allele effect on the association between *BTNL2 rs2076530 A* allele and sarcoidosis, multiple logistic regression was performed. The *rs2076530 AA* genotype (OR = 1.89, 95% CI = [1.17; 3.06], $p = 0.01$) remained significantly associated with disease risk. *DRB1*14* allele was still overrepresented in the patient group although not significantly so after adjusting for the *BTNL2* allele (OR = 3.29, 95% CI = [0.88; 12.21], $p = 0.08$). The effect modification was also tested in a regression model with terms for the *rs2076530 AA* genotype, *DRB1*14* allele and a cross-product interaction term. This interaction did not reach statistical significance.

Clinical presentation

When patients with ($n = 29$, 19.2%) and without ($n = 114$, 75.4%) Löfgren's syndrome were analyzed, we didn't find significant differences between *BTNL2 rs2076530 A* allele frequencies (Table 2). The association found between *BTNL2 rs2076530 A* allele and sarcoidosis was statistically significant for both groups: Löfgren's syndrome (OR = 2.09, 95% CI [1.88; 4.07], $p = 0.02$); non Löfgren's syndrome (OR = 1.56, 95% CI [1.08; 2.27], $p = 0.01$).

HLA-*DRB1*03* allele was also strongly associated with Löfgren's syndrome, both when compared with patients without Löfgren's syndrome (RR = 2.56; 95% CI = [1.62; 4.06], pc < 0.01) or controls (OR = 4.01, 95% CI = [1.88; 8.56], pc < 0.01). HLA-*DQB1*02* allele was also associated with Löfgren's syndrome patients, but LD between *DRB1*03* and *DQB1*02* alleles was statistically significant ($p < 0.001$). In a logistic regression model with HLA-*DRB1*03*, -*DQB1*02* and *rs2076530 A* alleles, only *DRB1*03* allele was significantly associated with Löfgren's syndrome, when compared with patients without Löfgren's syndrome.

The association of *DRB1*14* allele with sarcoidosis was only statistically significant for patients without Löfgren's syndrome (OR = 4.07, 95% CI = [1.0; 23.6]) with no statistically significant LD with *BTNL2 rs3076530* allele ($p = 0.22$), so this SNP remains associated with non-Löfgren's syndrome, after DRB1 adjustment.

Comparison of *rs206530* allele frequencies between patients with and without extra-thoracic involvement showed increased frequencies of A allele in those with isolated thoracic disease (71.5% vs 56.8%, RR = 1.32; 95% CI for RR = [1.05; 1.66]; $p = 0.01$), although with no significant differences among the radiological stages (data not shown). No differences were detected regarding HLA alleles between patients with isolated thoracic disease and those with extra-thoracic disease.

Table 1 BTNL2 and HLA alleles associated with sarcoidosis susceptibility.

Allele	Sarcoidosis	Controls	*p* value	pc	OR	95% CI
	n = 302 (%)[a]	*n* = 300 (%)[a]				
rs2076530 A	197 (65.2%)	167 (55.7%)	0.01	–	1.49	1.06–2.10
rs2076530 G	105 (34.8%)	133 (44.3%)	0.01	–	0.67	0.48–0.94
A*03	38 (12.6%)	23 (7.7%)	0.04	0.72	1.73	0.97–3.10
A*29	11 (3.6%)	23 (7.7%)	0.03	0.54	0.46	0.20–1.00
B*07	23 (7.6%)	11 (3.7%)	0.03	0.84	2.17	0.99–4.83
B*44	28 (9.3%)	52 (17.3%)	0.003	0.08	0.49	0.29–0.82
C*16	13 (4.3%)	34 (11.3%)	0.001	0.01	0.35	0.17–0.71
DRB1*07	33 (10.9%)	50 (16.7%)	0.04	0.52	0.61	0.37–1.01
DRB1*08	5 (1.7%)	17 (5.7%)	0.01	0.12	0.28	0.08–0.81
DRB1*14	11 (3.6%)	3 (1.0%)	0.03	0.39	3.74	0.97–21,06

[a] Allele frequencies; OR – Odds Ratio; CI – Confidence Interval; pc – corrected *p*-value for multiple comparisons.

Evolution

Comparison of *rs206530* allele frequencies between chronic patients and those who had disease resolution didn't show any statistically significant differences (Table 3). No differences were also seen comparing patients with resolution with those with chronic stable or progressive disease (data not shown). Moreover, comparison between these two groups of chronic patients didn't found any differences regarding *BTNL2 rs2076530 A* SNP (64.6% vs 62.2%, RR = 1.05; 95% CI for RR = [0.77; 1.44]; $p = 0.75$). However, regarding HLA alleles, disease resolution was significantly associated with *DRB1*03* (RR = 1.61; 95% CI = [1.23; 2.1]; $p = 0.004$).

Discussion

In this study, we evaluated a single nucleotide polymorphism of the *BTNL2* gene, as a putative genetic risk factor for sarcoidosis in a Portuguese population. The significant increase of the *BTNL2 rs2076530 A* allele observed, corroborates the role of this truncating SNP in disease susceptibility. Analyzing the subgroup presenting with Löfgren's syndrome, this association disappeared after HLA class II allele adjustment, since the determinant allele was HLA-*DRB1*03*; nevertheless, the association persists in the subgroup of patients without Löfgren syndrome even after adjustment for HLA-DRB1. An increased frequency of A allele was also noticed in those with isolated thoracic disease, although with no differences regarding radiological stages, disease resolution or a chronic course over at least 2 years of follow-up. HLA-*DRB1*03* allele, besides its association with Löfgren syndrome, was also significantly related with disease resolution.

BTNL2 is a member of the butyrophilin-like molecules (BTNLs) sharing structural homology with CD80/CD86 (B7) family of costimulatory molecules, expressed on antigen-presenting cells (APCs) and critical for effector immune responses.[12] BTNLs have a restricted expression in human cells and *BTNL2*, along with some epithelial cells of the mucosal barriers, is expressed on dendritic cells (spleen and lymph nodes)[13] and peripheral B cells,[14] consistent with a role in the modulation of APCs function. In fact, *BTNL2* has been shown to inhibit T cell proliferation and IL-2 production, also diminishing the production of pro-inflammatory cytokines in T cell cultures.[12–14] The single nucleotide polymorphism that we studied (*rs2076530 G > A*), results in a mutated *BTNL2* gene leading to a truncated protein and disruption of its membrane localization.[26] Although the precise functional implications of this mutation has not been established,[12] the putative inappropriate/inadequate membrane expression of a molecule inducing a negative signal to T-lymphocytes, could result in an uncontrolled high state of activation of T cells, a known pathological feature of sarcoidosis.[2,27,28]

One possible limitation of our study is the number of included cases. However, it represents the population currently referred to our university hospital center, which gives respiratory care to a total of 3 million inhabitants in the North region of Portugal (a country with 9 million inhabitants). This is a relatively homogeneous population and our controls were from the same geographic and genetic background. Another limitation concerns a sarcoidosis cohort in a tertiary care setting, potentially excluding less sever forms. The majority (59.7%) of our patients had

Table 2 BTNL2 and HLA class II alleles associated with Löfgren's syndrome (LS).

Allele	With LS	Without LS	*p*	pc	RR	95% CI
	n = 58 (%)[a]	*n* = 228 (%)[a]				
rs2076530 A	42 (72.4%)	151 (66.2%)	0.4	–	1.26	0.75–2.13
DRB1*03	16 (27.6%)	21 (9.2%)	0.0002	0.003	2.56	1.62–4.06
DRB1*04	1 (1.7%)	30 (13.2%)	0.01	0.13	0.14	0.02–1.01
DQB1*02	24 (41.4%)	42 (18.4%)	0.0002	0.001	2.35	1.51–3.67

[a] Allele frequencies; RR – relative risk; CI – confidence interval; pc – corrected *p*-value for multiple comparisons.

Table 3 BTNL2 and HLA class II alleles associated with disease outcome in sarcoidosis.

Allele	Resolution	Chronic	*p*	pc	RR	95% CI
	$n = 132$ (%)[a]	$n = 156$ (%)[a]				
rs2076530 A	91 (68.9%)	99 (63.5%)	0.32	–	1.14	0.87–1.51
DRB1*01	18 (13.6%)	10 (6.4%)	0.04	0.48	1.47	1.08; 2.00
DRB1*03	24 (18.2%)	11 (7.1%)	0.004	0.05	1.61	1.23–2.10

[a] Allele frequencies; RR – relative risk; CI – confidence interval; pc – corrected *p*-value for multiple comparisons.

isolated thoracic involvement, 19.2% Löfgren's syndrome, 43% disease resolution, 26.4% chronic stable disease and 25.1% chronic progressive disease, which seems in accordance with the usual presentations of Caucasians followed in specialized care settings.[1,2] Although not age matched to the patients, our controls were unrelated healthy individuals, recruited from the same geographic and ethnic background. Their comparatively higher mean age gives additional confidence, since a possible sarcoidosis diagnosis in their life time will be less likely.

Several studies have been published in different populations and ethnic groups, with different approaches regarding clinical phenotyping or HLA allele's adjustment.[11,15,16,29] Although with different conclusions, an overall evaluation of the available data supports a role of this SNP in the disease susceptibility. In our cohort there is also an unquestionable statistical significant relation between *BTNL2 rs2076530* SNP and sarcoidosis susceptibility. The consistency of these results is sustained by the fact that no linkage disequilibrium was found with *DRB1** alleles, namely *DRB1*14*, previously described as a risk allele in other sarcoidosis cohorts.[24,25]

Our findings are in accordance with the data from Caucasian populations. Rybicki et al. found a strong association of the *rs2076530* SNP with a white American cohort, but not in African American patients, although a three-locus haplotype that included *rs2076530* was associated with this population.[15] This study also suggested an influence of race in this genetic risk as the association with BTNL2 was independent of the HLA class II genes in whites but interacted antagonistically in African Americans, a population with greater genetic diversity. Valentonyte et al. also found an association of the *rs2076530 A* allele with predisposition to sarcoidosis in a white German population, with no apparent influence from HLA alleles.[11] Milman et al. found the A allele and an AA genotype significantly associated with sarcoidosis in a Danish population, although the degree of LD with DRB1 or DQB1 was not considered.[29]

On the other hand, Spagnolo et al. found a significant association between *BTNL2 rs2076530 A* allele on a cohort of United Kingdom and Netherlands patients, but the association disappeared after exclusion of Löfgren syndrome and adjusting for HLA-*DRB1* alleles.[16] Neither the study of Valentonyte et al. nor Rybicki et al. stratified their data according to Löfgren's syndrome,[11,15] a genetically and phenotypically different subset of patients characterized by an acute presentation and good prognosis.[1,2,6] Our results are somehow different from the British and Dutch cohort studied by Spagnolo et al., since the *BTNL2 rs2076530* SNP association was significant in the subgroup of patients without Löfgren syndrome, while the association with Löfgren syndrome patients in a univariate analysis was secondary to HLA-*DRB1*03* allele. Both a different genetic backgrounds and patient's selection may account for these discrepancies, as our patients were consecutively recruited from a tertiary reference center, a cohort more similar to the one reported by Valentonyte group.[11] Otherwise, the association of HLA-*DRB1*03* with Löfgren syndrome has been described in several populations[7,16,30–32] and different *DRB1*03* frequencies may even explain the rarity of this clinical presentation in Japan and the higher prevalence in Scandinavian countries.[6,33]

Interestingly, associations between *BTNL2 rs2076530 A* allele and a group of different diseases such as multiple sclerosis, leprosy, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus or Graves disease, were actually due to LD with other genes from this region, namely with various HLA-*DRB1* alleles, highlighting the difficulty to allocate primary associations in this highly dense gene area.[10,12,16,29] Thus, there is an absolute requirement for adjustment to HLA-*DR* alleles to every significant result related with *BTNL2* haplotypes, as we performed in our study.

Regarding the different sarcoidosis phenotypes, we also found a significant increase of *BTNL2 A* allele with isolated thoracic involvement. To our knowledge, this association has never been reported or addressed in previous studies, although no differences were seen among the four radiological Scadding stages, a finding also described by Spagnolo et al.[16]

Li et al. described for the first time an association between *BTNL2 rs2076530 A* SNP and a chronic disease course, in a German population.[18] However no linkage disequilibrium analysis to *DRB1* alleles was carried out, and the type of evolution of the chronic forms was not accessed. Coudurier et al. described the pathogenic variant of *BTNL2* on both alleles (*AA* homozygote) in three related patients, all with severe forms, although they also did not address HLA-*DR* linkage.[26] Collectively, these results do suggest an association of the variant allele with more severe cases with progression to fibrosis.

Generally we can consider three forms of evolution in sarcoidosis: disease resolution, chronic stable and chronic progressive disease (needing persistent therapeutic intervention).[19,34] In our cohort we stratified these different outcomes with the hypothesis that they might be related with different genetic risk factors. However, we didn't find any significant association between *BTNL2* and outcomes, neither with those who had disease resolution nor with who become chronic. Moreover, no differences between chronic stable or chronic progressive disease

were found. On the other hand, disease resolution was significantly associated with *DRB1*03*, an association also seen in various Caucasian populations.[30–32] In contrast with our observations, Wijnen et al. described that *BTNL2 rs2076530* variant allele was more frequent in patients with chronic persistent (progressive) disease than chronic non-persistent (stable) disease, independently from DRB1 allele's influence.[19] In their larger Dutch cohort, the A-allele almost doubled the risk of a progressive course of sarcoidosis.[19]

In conclusion, our study of a Portuguese sarcoidosis cohort at a tertiary reference centre showed a significant increase of the *BTNL2 rs2076530 A* allele frequency, with an even stronger association to the *BTNL2 rs2076530 AA* genotype, corroborating the role of this truncating SNP in the genetic risk of sarcoidosis in our population. Taking in account *BTNL2* and HLA-*DR* linkage disequilibrium, we also uncovered two independent genetic risk factors for different clinical presentations of sarcoidosis: the *BTNL2 rs2076530 A* allele in patients without Löfgren syndrome or with isolated thoracic disease, and the HLA-*DRB1*03* allele in those presenting Löfgren syndrome or disease resolution within 2 years of follow-up. The immunogenetic base of these associations deserves further investigation.

Conflict of interest statement

The authors have no conflict of interest.

References

1. Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. *N Engl J Med* 2007;**357**:2153–65.
2. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am J Respir Crit Care Med* 1999;**160**(2):736–55.
3. Grutters JC, Drent M, van den Bosh JMM. Diagnostic approach to sarcoidosis. In: du Bois R, Richeldi L, editors. *Interstitial lung diseases. European Respiratory Monograph* December 2009;vol. 46. p. 126–54. Chapter 9.
4. Baughman RP, Culver DA, Judson MA. A concise review of pulmonary sarcoidosis. *Am J Respir Crit Care Med* 2011;**183**:573–81.
5. Rybicki BA, Iannuzzi MC, Frederick MM, Thompson BW, Rossman MD, Bresnitz EA, Terrin ML, Moller DR, Barnard J, Baughman RP, DePalo L, Hunninghake G, Johns C, Judson MA, Knatterud GL, McLennan G, Newman LS, Rabin DL, Rose C, Teirstein AS, Weinberger SE, Yeager H, Cherniack R. Familial aggregation of sarcoidosis. A case-control etiologic study of sarcoidosis (ACCESS). *Am J Respir Crit Care Med* 2001;**164**: 2085–91.
6. Grunewald J. Review: role of genetics in susceptibility and outcome of sarcoidosis. *Semin Respir Crit Care Med* 2010;**31**:380–9.
7. Grunewald J, Brynedal B, Darlington P, Nisell M, Cederlund K, Hillert J, Eklund A. Different HLA-DRB1 allele distributions in distinct clinical subgroups of sarcoidosis patients. *Respir Res* 2010;**11**:25.
8. Müller-Quernheim J, Schürmann M, Hofmann S, Gaede KI, Fischer A, Prasse A, Zissel G, Schreiber S. Genetics of sarcoidosis. *Clin Chest Med* 2008;**29**:391–414.
9. Iannuzzi MC, Rybicki BA. Genetics of sarcoidosis: candidate genes and genome scans. *Proc Am Thorac Soc* 2007;**4**:108–16.
10. Grunewald J. Genetics of sarcoidosis. *Curr Opin Pulm Med* 2008;**14**:434–9.
11. Valentonyte R, Hampe J, Huse K, Rosenstiel1 P, Albrecht M, Stenzel A, Nagy M, Gaede KI, Franke A, Haesler R, Koch A, Lengauer T, Seegert D, Reiling N, Ehlers S, Schwinger E, Platzer M, Krawczak M, Müller-Quernheim J, Schürmann M, Schreiber S. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. *Nat Genet* 2005;**37**:357–64.
12. Arnett HA, Escobar SS, Viney JL. Regulation of costimulation in the era of butyrophilins. *Cytokines* 2009;**46**:370–5.
13. Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, et al. BTNL2, a butyrophilin/B7-like molecule, is a negative costimulatory molecule modulated in intestinal inflammation. *J Immunol* 2007;**178**:1523–33.
14. Nguyen T, Liu XK, Zhang Y, Dong X. BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation. *J Immunol* 2006;**176**:7354–60.
15. Rybicki BA, Walewski JL, Maliarik MJ, Kian H, Iannuzzi MCThe ACCESS Research Group. The BTNL2 gene and sarcoidosis susceptibility in African Americans and Whites. *Am J Hum Genet* 2005;**77**:491–9.
16. Spagnolo P, Sato H, Grutters JC, Renzoni EA, Marshall SE, Ruven HJT, et al. Analysis of BTNL2 genetic polymorphisms in British and Dutch patients with sarcoidosis. *Tissue Antigens* 2007;**70**:480–6.
17. Mochida A, Kinouchi Y, Negoro K, Takahashi S, Takagi S, Nomura E, et al. Butyrophilin-like 2 gene is associated with ulcerative colitis in the Japanese under strong linkage disequilibrium with HLA-DRB1_1502. *Tissue Antigens* 2007;**70**:128–35.
18. Li Y, Wollnik B, Pabst S, Lennarz M, Rohmann E, Gillissen A, et al. BTNL2 gene variant and sarcoidosis. *Thorax* 2006;**61**:273–4.
19. Wijnen PA, Voorter CE, Nelemans PJ, Verschakelen JA, Bekers O, Drent M. Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression? *Hum Immunol* 2011;**72**:342–7.
20. Scadding JG. Prognosis of intrathoracic sarcoidosis in England: a review of 136 cases after 5 years' observation. *BMJ* 1961;**2**: 1165–72.
21. Judson MA, Baughman RP, Thompson BW, Teirstein AS, Terrin ML, Rossman MD, Yeager Jr H, McLennan G, Bresnitz EA, DePalo L, Hunninghake G, Iannuzzi MC, Johns CJ, Moller DR, Newman LS, Rabin DL, Rose C, Rybicki BA, Weinberger SE, Knatterud GL, Cherniack R. Two year prognosis of sarcoidosis: the ACCESS experience. *Sarcoidosis Vasc Diffuse Lung Dis* 2003; **20**(3):204–11.
22. Excoffier L, Laval G, Schneider S. Arlequin version 3.0: an integrated software package for population genetics data analysis. *Evol Bioinform Online* 2005;**1**:47–50.
23. Spínola H, Middleton D, Brehm A. HLA genes in Portugal inferred from sequence-based typing: in the crossroad between Europe and Africa. *Tissue Antigens* 2005;**66**:26–36.
24. Foley PJ, McGrath DS, Puscinska E, Petrek M, Kolek V, Drabek J, Lympany PA, Pantelidis P, Welsh KI, Zielinski J, du Bois RM. Human leukocyte antigen-DRB1 position 11 residues are a common protective marker for sarcoidosis. *Am J Respir Cell Mol Biol* 2001;**25**:272–7.
25. Grutters J, Sato H, Welsh K, du Bois RM. The importance of sarcoidosis genotype to lung phenotype. *Am J Respir Cell Mol Biol* 2003;**29**:S59–62.
26. Coudurier M, Freymond N, Aissaoui S, Calender A, Pacheco Y, Devouassoux G. Homozygous variant rs2076530 of BTNL2 and familial sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2009;**26**: 162–6.
27. Bargagli E, Bianchi N, Margollicci M, Olivieri C, Luddi A, Coviello G, Grosso S, Rottoli P. Chitotriosidase and soluble IL-2 receptor: comparison of two markers of sarcoidosis severity. *Scand J Clin Lab Invest* 2008;**68**(6):479–83.

28. Facco M, Baesso I, Miorin M, Bortoli M, Cabrelle A, Boscaro E, Gurrieri C, Trentin L, Zambello R, Calabrese F, Cassatella MA, Semenzato G, Agostini C. Expression and role of CCR6/CCL20 chemokine axis in pulmonary sarcoidosis. *J Leukoc Biol* 2007; **82**(4):946—55.
29. Milman N, Svendsen CB, Nielsen FC, Hansen TO. The BTNL2 A allele variant is frequent in Danish patients with sarcoidosis. *Clin RespirJ* 2011;**5**:105—11.
30. Morais A, Alves H, Lima B, Delgado L, Goncalves R, Tafulo S. HLA class I and II and TNF-alpha gene polymorphisms in sarcoidosis patients. *Rev Port Pneumol* 2008;**14**:727—46.
31. Martinetti M, Tinelli C, Kolek V, et al. ''The sarcoidosis map'': a joint survey of clinical and immunogenetic findings in two European countries. *Am J Respir Crit Care Med* 1995;**152**:557—64.
32. Bogunia-Kubik K, Tomeczko J, Suchnicki K, Lange A. HLA-DRB1*03, DRB1*11 or DRB1*12 and their respective DRB3 specificities in clinical variants of sarcoidosis. *Tissue Antigens* 2001;**57**:87—90.
33. Morimoto T, Azuma A, Abe S, Usuki J, Kudoh S, Sugisaki K, Oritsu M, Nukiwa T. Epidemiology of sarcoidosis in Japan. *Eur Respir J* 2008;**31**:372—9.
34. Paramothayan S, Lasserson T. Treatments for pulmonary sarcoidosis. *Respir Med* 2008;**102**:1—9.

Annexin A11 gene polymorphism (R230C variant) and sarcoidosis in a Portuguese population

António Morais, Bruno Lima, Maria Peixoto, Natália Melo, Helena Alves, J. Agostinho Marques, Luís Delgado

Tissue Antigens, 2013, 82, 186–191

TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

Tissue Antigens ISSN 0001-2815

Annexin A11 gene polymorphism (R230C variant) and sarcoidosis in a Portuguese population

A. Morais[1,2], B. Lima[3], M. Peixoto[3], N. Melo[1], H. Alves[3], J. A. Marques[1,2] & L. Delgado[4,5]

1 Department of Pneumology, Hospital São João, Porto, Portugal
2 Department of Medicine, Faculdade de Medicina do Porto, Porto, Portugal
3 North Histocompatibility Centre, Porto, Portugal
4 Immunology Lab, Faculdade de Medicina do Porto, Porto, Portugal
5 Center for Research in Health Technology and Information Systems (CINTESIS), Faculdade de Medicina do Porto, Porto, Portugal

Key words
ANXA11; evolution; Löfgren syndrome; *rs1049550* single nucleotide polymorphism

Correspondence
António Morais
Department of Pneumology
Hospital São João/ Faculdade de Medicina do Porto
Alameda Professor Hernani Monteiro
Porto 4200-319
Portugal
Tel: +351919175999
Fax: +351225503338
e-mail: amorais3@hotmail.com

Received 9 May 2013; revised 22 July 2013; accepted 22 July 2013

doi: 10.1111/tan.12188

Abstract

A recent genome-wide association study detected a protective effect for the annexin A11 *rs1049550**T allele (R230Cvariant) in susceptibility to sarcoidosis. We evaluated the association between *rs1049550* C/T and sarcoidosis susceptibility, distinct disease phenotypes and evolution in a Portuguese population. We performed a case–control study of 208 patients and 197 healthy controls. Samples were genotyped for *rs1049550* C/T using real-time polymerase chain reaction. The frequency of the annexin A11 *rs1049550**T allele was significantly lower in patients than in controls (33.2 *vs* 44.9%, $P < 0.001$). Odds ratio of 0.52 and 0.44 were obtained, respectively for carriers of one (CT) and two (TT) copies normalized to the CC wild-type genotype ($P < 0.001$). There were no significant differences in patients with and without Löfgren syndrome. A significant increase in the frequency of the T allele was observed in patients with bronchoalveolar lavage (BAL) fluid neutrophilia ($P = 0.04$). No significant associations were seen for lung function pattern, radiological stages or different forms of disease evolution. Our study confirms that *rs1049550**T allele exerts a significant protective effect on sarcoidosis susceptibility. Given the role of annexin A11 in cell division, apoptosis and neutrophil function, this polymorphism may affect key elements of granulomatous and interstitial inflammation in sarcoidosis.

Introduction

Sarcoidosis is a multisystemic disorder of unknown etiology, characterized by the formation of noncaseating granulomas, predominantly in the lungs and lymph nodes (1, 2). Interaction between a multitude of genes and unknown environmental factors seems to be the most plausible explanation for the pathogenesis of this disorder (1, 2). Disease clustering in families and different racial and ethnic incidence rates and clinical presentations lend support to the theory that genetics plays a determining role in disease susceptibility and heterogeneity (3).

The strongest and most consistent association described so far is related to human leukocyte antigen (HLA) haplotypes, namely HLA class II alleles, which have been linked to both disease susceptibility and phenotype expression (4–6). However, there is a high degree of linkage disequilibrium within the major histocompatibility complex (MHC) and it has not yet been clarified whether sarcoidosis susceptibility is directly influenced by HLA genes or whether the associations observed are due to other genes in linkage disequilibrium with this region (6, 7). This is the case of the butyrophilin-like 2 gene (*BTNL2*), located at the junction of the HLA class II and class III regions which has been associated with susceptibility to sarcoidosis in different populations and phenotypes (3, 8–10).

Recently, Hofmann et al. (11), in a genome-wide search for unknown genetic mutations associated with sarcoidosis evaluated more than 440,000 single nucleotide polymorphisms (SNPs) in a population of 499 German patients and 500 controls. The study showed that the strongest association was with a SNP (*rs1049550* C/T) in the annexin A11 gene at chromosome 10q22.3. Meanwhile, this finding was replicated in Czech and American populations (12, 13). Annexin A11 is a member of the annexin family of calcium-dependent phospholipid-binding proteins (14). Although the functional consequences of *rs1049550* C/T SNP have not been entirely elucidated, a depletion or dysfunction of annexin A11 may affect the apoptosis pathway, inducing an imbalance between apoptosis and survival of activated inflammatory cells (14, 15).

The aim of this study was to evaluate the SNP *rs1049550* C/T (R230C variant) as a putative genetic marker for sarcoidosis in a Portuguese population and to explore its association with clinically distinct disease phenotypes and evolution.

Materials and methods

Study power

For a case–control study with 1:1 ratio, a type 1 error rate of 5%, an 80% of power to detect a modest genotype odds ratio (OR) of 0.65 for disease susceptibility with an expected frequency of exposure in control group of 45% (12, 13, 16), we estimated a sample size with 187 patients and 187 controls. We included 208 unrelated Caucasian patients with a mean ± standard deviation age of 38.3 ± 12 years; 58.7% ($n = 122$) were women.

Patients

All attended the Interstitial Lung Diseases outpatient clinic at Centro Hospitalar São João, a tertiary referral center serving patients mostly from the Oporto district and the north of Portugal. All the patients had thoracic sarcoidosis, as determined by symptoms, radiology and lung function tests, and supported by evidence of noncaseating epithelioid cell granulomas in biopsy specimens in 69% of cases. All individuals without histological confirmation fulfilled the criteria in the consensus statement on sarcoidosis by the European Respiratory Society (ERS), the American Thoracic Society (ATS) and the World Association of Sarcoidosis and Other Granulomatous Diseases (WASOG) (1), namely compatible clinical and radiographic features, a BAL fluid CD4/CD8 lymphocyte ratio of >4.0, and a 2-year observation period to exclude other medical conditions. Löfgren syndrome was defined as the presence of bilateral hilar lymphadenopathy, fever, ankle arthralgia and erythema nodosum (1). Regular smoking habits were expressed by 12.2% of the patients and 13% were ex-smokers. Lung function tests were performed and results classified as normal, obstructive, restrictive or mixed pattern according to the ERS/ATS Task Force criteria (17). Thoracic involvement was classified according to the Scadding criteria (stage 0 – no thoracic involvement, stage I – adenopathies without lung involvement, stage II – adenopathies and lung involvement, stage III – lung involvement only and stage IV – lung fibrosis) (1). BAL was performed according to ERS recommendations, with appropriate processing and interpretation of specimens (18).

Disease resolution was defined as the disappearance of symptoms and normalization of chest x-ray and lung function tests within 2 years of diagnosis (1, 19) Patients with evidence of disease after 2 years were considered to have chronic sarcoidosis. Chronic disease was further classified as chronic stable (no disease progression and/or no need for medication) or chronic unstable (disease progression and/or need for continuous medication) (20, 21). A control group was formed by 197 randomly selected unrelated healthy volunteer bone marrow donors from the same geographic region (22). Their mean age was 31.8 ± 7 years and 62.4% were women. None had a history of lung disease or had respiratory symptoms or evidence of any other disease by chest radiography. Written consent was obtained from all individuals and the study was approved by the hospital ethics committee.

Genotyping

Genomic DNA from patients and controls was extracted from peripheral whole blood samples and collected in acid citrate dextrose (ACD) anticoagulant tubes, using either QIAsymphony or QIAmp DNA kits (Qiagen, Venlo, The Netherlands). DNA samples were genotyped for rs1049550 C/T (R230C) using a TaqMan SNPs genotyping assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Minor groove binding probes were labeled with the fluorescent dyes fluorescein amidite (FAM) and VIC. Polymerase chain reaction was performed in a total reaction volume of 12.5 Kl with TaqMan Genotyping Master Mix 1×, 20 ng of genomic DNA and the SNP genotyping assay 1×. The amplification protocol began with a denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and annealing and extension at 60°C for 1 min. After polymerase chain reaction, the genotype of each sample was automatically determined by measuring allelic-specific fluorescence on the ABI PRISM 7000 Sequence Detection Systems using SDS 1.2 software for allelic discrimination (Applied Biosystems).

Statistical analysis

Allele and genotype distributions were statistically analyzed with the chi-squared test, or the Fisher exact test when appropriate. Relative risks or ORs with their 95% confidence intervals (CIs) were calculated as association measures. The software used was the STATCALC program (EpiInfo 2002, Centers for Disease Control and Prevention, Atlanta, GA). *P*-values of less than 0.05 were considered statistically significant. Annexin A11 allele frequencies in patients and controls were determined for each allele using the following formula: allele frequency (%) = $(n/2N) \times 100$, where n indicates the sum of a particular allele and N indicates the total number of individuals. Deviations from the Hardy–Weinberg equilibrium were tested with ARLEQUIN v3 software (23) using the method described by Guo and Thompson (24).

Results

The distribution of *rs1049550* genotypes did not show any significant departure from the Hardy–Weinberg equilibrium, *P*-values for the exact test exceeded 0.05 in both

Table 1 Association between annexin A11 *rs1049550 C/T* single nucleotide polymorphism and susceptibility to sarcoidosis[a]

	Sarcoidosis	Controls	OR	95% CI	*P*-value
Allele					
rs1049550 C	278 (66.8%)[b]	217 (55.1%)[b]	1		
rs1049550 T	138 (33.2%)[b]	177 (44.9%)[b]	0.61	0.45–0.82	<0.001
Total (2*n*)	416	394			
Genotype					
CC	99 (47.6%)	61 (31.0%)	1		0.001
CT	80 (38.5%)	95 (48.2%)	0.52		
TT	29 (13.9%)	41 (20.8%)	0.44		
Total (*n*)	208	197			

CI, confidence interval; OR, odds ratio.
[a]Allele frequencies of annexin A11 variants between patients and controls were compared using the chi-squared test; genotype frequencies were compared using the chi-squared test for trend normalized to the CC homozygotes (OR set to 1).
[b]Allele frequencies.

Table 2 Frequency of annexin A11 *rs1049550 C/T* single nucleotide polymorphism in patients with sarcoidosis according to presence or absence of Löfgren syndrome (LS)[a]

	Sarcoidosis			Sarcoidosis without LS *vs* controls			Sarcoidosis witht LS *vs* controls		
	With LS	Without LS	Controls	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Allele									
rs1049550 C	70 (64%)[b]	198 (68%)[b]	217 (55.1%)[b]	1		0.001	1		0.11
rs1049550 T	40 (36%)[b]	92 (32%)[b]	177 (44.9%)[b]	0.57	0.41–0.79		0.76	0.53–1.07	
Total (2*n*)	110	290	394						
Genotype									
CC	25 (46%)	71 (49%)	61 (31.0%)	1		0.001	1		0.12
CT	20 (36%)	56 (39%)	95 (48.2%)	0.51			0.51		
TT	10 (18%)	18 (12%)	41 (20.8%)	0.38			0.6		
Total (*n*)	55	145	197						

CI, confidence interval; OR, odds ratio.
[a]Allele and genotype frequencies of annexin A11 variants between patients and controls were compared using the chi-squared test; genotype frequencies were compared using the chi-squared test for trend normalized to the CC homozygotes (OR set to 1). Eight patients were not included because the presence of Löfgren syndrome could not be confirmed.
[b]Allele frequencies.

patients and healthy controls groups. The frequency of the *rs1049550**T allele was significantly lower in sarcoidosis patients (33.2%) than in controls (44.9%) ($P < 0.001$, OR = 0.61, 95% CI = 0.45–0.82). The frequencies of annexin A11 alleles are summarized in Table 1. An OR of 0.52 and 0.44 for sarcoidosis was obtained, respectively, for the carriers of one (genotype CT) and two (genotype TT) copies of the *rs1049550**T allele, when compared with the CC wild-type genotype ($P < 0.001$). When the patients were divided into subgroups with ($n = 55$) and without ($n = 145$) Löfgren syndrome, we observed no significant differences in the frequency of the *rs1049550**T allele ($P = 0.37$). However, when these subgroups were compared with controls, only those without Löfgren syndrome had a significant decreased frequency of the allele (Table 2). No significant differences were observed between frequencies of *rs1049550*T* when patients were compared in terms of lung function impairment patterns and radiological stages (Scadding criteria). We did, however, observe a significant increase in the frequency of the T allele in patients with neutrophilia in BAL fluid (>3%; $n = 30$) (45 *vs* 31%, $P = 0.04$, OR = 1.58, 95%CI = 1.02–2.47) and this association persisted after adjustment to smoking habits and the presence of lung fibrosis. No significant associations were found between the presence of the *rs1049550**T allele and disease progression (resolution or chronicity) over a period of either 2 or 5 years (Table 3) or when the chronic stable ($n = 41$) or chronic unstable ($n = 42$) forms of the disease were compared (data not shown).

Discussion

In this study, we evaluated a SNP within annexin A11, a member of the annexin family of calcium-dependent phospholipid-binding proteins located at chromosome 10q22.3, as a putative genetic protective factor for sarcoidosis in a Portuguese population. We observed a significant decrease in the frequency of the annexin A11 *rs1049550**T allele in this population, which supports a role of this SNP in susceptibility to sarcoidosis.

Table 3 Frequency of annexin A11 *rs1049550 C/T* single nucleotide polymorphism in sarcoidosis according to disease resolution[a]

	Chronicity (5 years)[b]	Resolution	*P*-value
Allele			
rs1049550 C	74 (60%)[c]	114 (66%)[c]	0.3
rs1049550 T	50 (40%)[c]	58 (34%)[c]	
Total (2*n*)	124	172	
Genotype			
CC	25 (40%)	39 (45%)	0.3
CT	24 (39%)	36 (42%)	
TT	13 (21%)	11 (13%)	
Total (*n*)	62	86	

[a]Allele and genotype frequencies of annexin A11 variants between group of patients were performed using the chi-squared test.
[b]Chronic forms were considered when there was evidence of disease after 5 years of follow-up.
[c]Allele frequencies.

Yet, when we analyzed subgroups of patients with different phenotypes, such as Löfgren syndrome, the association was maintained only in patients without this syndrome. No significant associations were found for other clinical presentations, namely different radiological stages of sarcoidosis or disease progression, although the frequency of the annexin A11 TT genotype was higher in sarcoidosis patients with increased BAL fluid neutrophil counts.

Hofman et al. (11) using a genome-wide search, were the first group to report a strong association between sarcoidosis and the SNP *rs1049550* C/T at chromosome 10q22.3 in a group of German patients. Although a strong association was detected for different SNPs, including several intronic and intergenic SNPs (*rs1953600*, *rs2573346*, *rs2784773* and *rs2789679*), they were all in strong linkage disequilibrium with *rs1049550*. Subsequently, Li et al. (16) confirmed the association of *rs1049550* with sarcoidosis susceptibility in a cohort of 325 patients, also of German origin, in comparison with 364 controls (35 *vs* 45%). The group also reported a strong association for *rs2573346* but they did not assess linkage disequilibrium. Posteriorly Mrazek et al. (12). found the same association between *rs1049550* C/T and sarcoidosis risk in a group of 245 Czech patients, as compared with 254 healthy controls, with a significantly lower frequency of the *rs1049550**T allele in patients (35%) compared with controls (42%). More recently, Levin et al. (13), in a search for additional annexin 11 variants independently associated with sarcoidosis in a North American population, also described a significantly decreased frequency of the sarcoidosis-related *rs1049550**T allele in 447 European Americans (36 *vs* 42% in controls) and in 1242 African Americans (15 *vs* 17% in controls), suggesting a cross-ethnic influence of this SNP. After adjustment for *rs1049550*, the authors found two additional associations with sarcoidosis susceptibility – *rs61860052* and *rs4377299* – but only in the African-American subgroup. The results of this study, conducted in a European Caucasian population but with a different genetic background (25), are very similar, with *rs1049550**T allele frequencies of 33.2% in patients and 44.9% in healthy controls, providing support for the existence of an association between this allele and sarcoidosis. In line with the findings of previous studies, the protective effect of the *rs1049550**T allele increased with the number of copies in the genotype (gene-dose effect, Figure 1 and Table S1).

Annexin A11 is expressed in a wide range of tissues and immune cells, with high expression patterns detected in thymus, lung and smooth muscle, suggesting its involvement in basic cellular processes that are essential to all cells (11, 26). It consists of an N-terminal proline-tyrosine-glycine (PYG)-rich region, followed by annexin core domains (four annexin repeats) in the C-terminal region of the molecule, responsible for its calcium-binding properties (26). The mutation identified in the genome-wide study by Hofmann et al. (11) is localized in exon 6 and results in the substitution of a cytosine with a thymidine, which alters the codon from a basic arginine to a polar cysteine at residue 230 (R230C), within the initial 14 residues of the first annexin repeat (26, 27). This SNP had previously been detected in a bioinformatic analysis of non-synonymous mutations in over 5500 human genes, and was noted as a SNP likely to have a deleterious effect (e.g. the sequence changes are likely to either disrupt the structure of the protein or interfere with the function or interactions of the protein) (28).

The functional significance of *rs1049550* C/T and the possible mechanism of action of annexin A11 in sarcoidosis have yet to be elucidated (27, 29). Several studies have shown annexin A11 to play a role in the regulation of cell proliferation and apoptosis (12, 27). Annexin A11 binds calcyclin and ALG-2, which are implicated in regulating apoptosis in caspase-dependent and independent pathways, respectively (30). Moreover, small interface siRNA-mediated depletion of annexin A11 results in the absence of a midbody and impaired cell division (29, 31). It is possible that these cytokinetic defects and apoptosis deregulation contribute to the formation of multinucleate cells and persistent inflammation, affecting granuloma formation or its resolution in sarcoidosis (32).

Taking into consideration different types of disease presentation, Mrazek et al. (12) found that *rs1049550* was significantly associated with the presence of isolated lymphadenopathy compared with pulmonary involvement in patients with sarcoidosis. Moreover, they found that TT homozygotes were three times more frequent in patients with Löfgren syndrome (21%) than in those without (7%). Our results differed as we only saw significant differences in annexin A11 SNP frequencies in the subgroup of patients without Löfgren syndrome as compared with controls. This is somewhat in agreement with the data of Li et al. (16), who did not find any differences between acute forms of sarcoidosis (which almost certainly included Löfgren syndrome) and

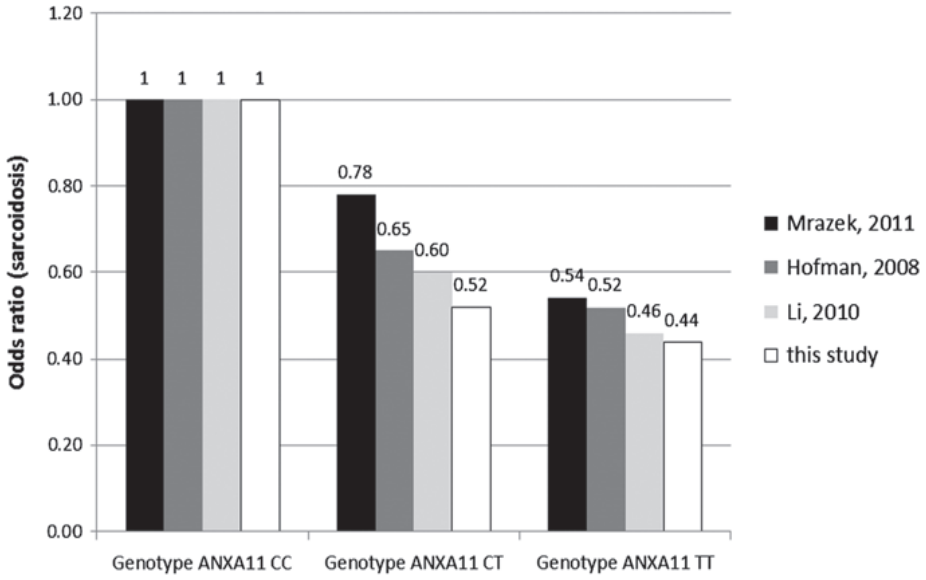


Figure 1 Estimated odds ratio for the protective effect of the annexin A11 rs1049550*T allele in sarcoidosis patients according to whether they are carriers of one (genotype ANXA11 CT) or two (genotype ANXA11 TT) copies normalized to the CC homozygotes (odds ratio set to 1). The gene-dose effect for sarcoidosis was estimated by the chi-squared test for trend. Results are extrapolated from data included in references (11, 12, 16).

chronic forms. Moreover, Levin et al. (13) found a significant association between *rs4399277* and Scadding stage IV disease in an African-American subgroup. In our study no differences were found when we compared stage I or stage IV sarcoidosis with other Scadding stages.

In our study, the frequency of the annexin A11 TT genotype was increased in patients with increased BAL fluid neutrophil counts. Annexin A11 is secreted by activated neutrophils and its cytoplasmatic expression changes during phagocytosis (33–35). It is therefore possible that annexin A11 participates in neutrophil function (in addition to apoptosis regulation), an area deserving further investigation. Although BAL fluid neutrophilia in sarcoidosis has been associated with more severe disease (36, 37), we did not find any significant associations between the annexin A11 SNP and disease progression (resolution or chronic stable or unstable disease). Li et al. (16) also investigated an association with disease progression (over 2 years), and found no differences between acute and chronic forms, which were both statistically associated either with *rs1049550* or *rs257334*. Some authors have suggested that a 5-year period is more appropriate for ascertaining an accurate outcome. We considered both 2 and 5 years but did not observe any significant associations for the annexin A11 *rs1049550* C/T SNP.

In conclusion, our study confirms the strong association and protective effect of the annexin A11 *rs1049550* C/T SNP in susceptibility to sarcoidosis in a Caucasian Portuguese population. Although the functional significance of this SNP is still unknown, given the role of annexin A11 in cell division, apoptosis and neutrophil function, it may affect key elements of the granulomatous and interstitial inflammation that characterizes sarcoidosis. Further studies are needed to clarify these hypotheses.

Conflict of Interests

The authors confirm that there are no conflicts of interest.

References

1. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am J Respir Crit Care Med* 1999: **160**: 736–55.
2. Spagnolo P, Luppi F, Roversi P, Cerri S, Fabbri LM, Richeldi L. Sarcoidosis: challenging diagnostic aspects of an old disease. *Am J Med* 2012: **125**: 118–25.
3. Grunewald J. Review: role of genetics in susceptibility and outcome of sarcoidosis. *Semin Respir Crit Care Med* 2010: **31**: 380–9.
4. Grunewald J, Brynedal B, Darlington P et al. Different HLA-DRB1 allele distributions in distinct clinical subgroups of sarcoidosis patients. *Respir Res* 2010: **11**: 25.
5. Morais A, Alves H, Lima B, Delgado L, Goncalves R, Tafulo S. HLA class I and II and TNFalpha gene polymorphisms in sarcoidosis patients. *Rev Port Pneumol* 2008: **14**: 727–46.

6. Iannuzzi MC, Rybicki BA. Genetics of sarcoidosis: candidate genes and genome scans. *Proc Am Thorac Soc* 2007: **4**: 108–16.
7. Grunewald J. Genetics of sarcoidosis. *Curr Opin Pulm Med* 2008: **14**: 434–9.
8. Valentonyte R, Hampe J, Huse K et al. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. *Nat Genet* 2005: **37**: 357–64.
9. Spagnolo P, Sato H, Grutters JC et al. Analysis of BTNL2 genetic polymorphisms in British and Dutch patients with sarcoidosis. *Tissue Antigens* 2007: **70**: 480–6.
10. Morais A, Lima B, Peixoto MJ, Alves H, Marques A, Delgado L. BTNL2 gene polymorphism associations with susceptibility and phenotype expression in sarcoidosis. *Respir Med* 2012: **106**: 1771–7.
11. Hofmann S, Franke A, Fischer A et al. Genome-wide association study identifies ANXA11 as a new susceptibility locus for sarcoidosis. *Nat Genet* 2008: **40**: 1103–6.
12. Mrazek F, Stahelova A, Kriegova E et al. Functional variant ANXA11 R230C: true marker of protection and candidate disease modifier in sarcoidosis. *Genes Immun* 2011: **12**: 490–4.
13. Levin AM, Iannuzzi MC, Montgomery CG et al. Association of ANXA11 genetic variation with sarcoidosis in African Americans and European Americans. *Genes Immun* 2012: **12**: 67–77.
14. Gerke V, Moss SE. Annexins: from structure to function. *Physiol Rev* 2002: **82**: 331–71.
15. Farnaes L, Ditzel HJ. Dissecting the cellular functions of annexin XI using recombinant human annexin XI-specific autoantibodies cloned by phage display. *J Biol Chem* 2003: **278**: 33120–6.
16. Li Y, Pabst S, Kubisch C, Grohe C, Wollnik B. First independent replication study confirms the strong genetic association of ANXA11 with sarcoidosis. *Thorax* 2010: **65**: 939–40.
17. ERS/ATS Task Force. Report on standardization of lung function testing. *Eur Respir J* 2005: **28**: 319–38.
18. European Society of Pneumology Task Group. Report on technical recommendations and guidelines for bronchoalveolar lavage (BAL). *Eur Respir J* 1989: **2**: 561–85.
19. Judson MA, Baughman RP, Thompson BW et al. Two year prognosis of sarcoidosis: the ACESS experience. *Sarcoidosis Vasc Diffuse Lung Dis* 2003: **20**: 204–11.
20. Baughman RP, Nagai S, Balter M et al. Defining the clinical outcome status (COS) in sarcoidosis: results of WASOG Task Force. *Sarcoidosis Vasc Diffuse Lung Dis* 2011: **28**: 56–64.
21. Wijnen PA, Voorter CE, Nelemans PJ, Verschakelen JA, Bekers O, Drent M. Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression? *Hum Immunol* 2011: **72**: 342–7.
22. Lima BA. Bone marrow volunteer donors recruitment in Northern Portugal. *Acta Med Port* 2011: **24**: 301–6.
23. Excoffier L, Schneider S. Arlequin ver 3.0: an integrated software package for population genetics data analysis. *Evol Bioinform Online.* 2005: **1**: 47–50.
24. Guo S, Thompson E. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 1992: **48**: 361–72.
25. Lima BA, Alves H. HLA-A, -C, -B, and -DRB1 allelic and haplotypic diversity in bone marrow volunteer donors from Northern Portugal. *Organs, Tissues & Cells* 2013: **16**: 19–26.
26. Mizutani A, Usuda N, Tokumitsu H et al. CAP-50, a newly identified annexin, localizes in nuclei of cultured fibroblast 3Y1 cells. *J Biol Chem* 1992: **267**: 13498–504.
27. Fatimathas L, Moss SE. Characterisation of the sarcoidosis-associated variant of annexin A11. *Gen Physiol Biophys* 2009: **28** (Spec No Focus): F29–38.
28. Burke DF, Worth CL, Priego EM et al. Genome bioinformatic analysis of nonsynonymous SNPs. *BMC Bioinformatics* 2007: **8**: 301.
29. Fatimathas L, Moss SE. Annexins as disease modifiers. *Histol Histopathol* 2010: **4**: 527–32.
30. Vito P, Lacana E, D'Adamio L. Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science* 1996: **271**: 521–5.
31. Tomas A, Futter C, Moss SE. Annexin 11 is required for midbody formation and completion of the terminal phase of cytokinesis. *J Cell Biol* 2004: **165**: 813–22.
32. Kuriyama R, Gustus C, Terada Y, Uetake Y, Matuliene J. CHO1, a mammalian kinesin-like protein, interacts with F-actin and is involved in the terminal phase of cytokinesis. *J Cell Biol* 2002: **156**: 783–90.
33. Sjolin C, Dahlgren C. Isolation by calcium-dependent translation to neutrophil specific granules of a 42-kD cytosolic protein, identified as being a fragment of annexin XI. *Blood* 1996: **87**: 4817–23.
34. Sjolin C, Movitz C, Lundqvist H, Dahlgren C. Translocation of annexin XI to neutrophil subcellular organelles. *Biochim Biophys Acta* 1997: **1326**: 149–56.
35. Boussac M, Garin J. Calcium-dependent secretion in human neutrophils: a proteomic approach. *Electrophoresis* 2000: **21**: 665–72.
36. Drent M, Jacobs JA, de Vries J, Lamers R, Liem I, Wouters E. Does the cellular bronchoalveolar lavage fluid profile reflect the severity of sarcoidosis? *Eur Respir J* 1999: **13**: 1338–44.
37. Ziegenhagen MW, Rothe ME, Schlaak M, Müller-Quernheim J. Bronchoalveolar and serological parameters reflecting the severity of sarcoidosis. *Eur Respir J* 2003: **21**: 407–13.

Supporting Information

The following supporting information is available for this article:

Table S1. Comparison of the distribution of ANXA11 rs1049550 genotypes and alleles between the groups of patients with sacoidosis and control subjects in Portuguese (this study), Czech (12), German (11,16) populations.

Associations between sarcoidosis clinical course and ***ANXA11 rs1049550*** C/T, ***BTNL2 rs2076530*** G/A, and HLA class I and II alleles

António Morais, Bruno Lima, Helena Alves, Natália Melo, Patrícia Mota, Agostinho Marques, Luís Delgado

BMC Pulmonary Medicine, submetido, em Revisão, 2015

Associations between sarcoidosis clinical course and *ANXA11 rs1049550* C/T, *BTNL2 rs2076530* G/A, and HLA class I and II alleles

António Morais[1,2], Bruno Lima[3], Helena Alves[4], Natália Melo[1], Patricia Mota[1,2], Agostinho Marques[1,2], Luis Delgado[5]

1- Serviço de Pneumologia, Centro Hospitalar São João, Porto, Portugal
2- Faculdade de Medicina, Universidade do Porto, Portugal
3- Oficina de Bioestatistica, Ermesinde, Portugal
4- Unidade da Promoção da Saúde e Prevenção de Doenças Não Transmissíveis. Instituto Nacional de Saúde (INSA) Dr. Ricardo Jorge, Porto, Portugal
5- Immunology Lab, Basic & Clinical Immunology Unit, Faculty of Medicine, and Center for Health Technology and Services Research (CINTESIS), University of Porto, Portugal

Abstract

A genetic background may be responsible for the different clinical courses in sarcoidosis.

We analyzed associations between sarcoidosis clinical course and HLA class I/II alleles and susceptibility gene SNPs *ANXA11 rs1049550* C/T and *BTNL2 rs2076530* G/A in a Portuguese population, investigating possible gene-gene interactions.

We studied 138 unrelated Caucasian sarcoidosis patients (78 women, 56.5%; mean age, 37.2±12.1 years). Disease that persisted after 2 years was considered chronic. Samples were genotyped for *ANXA11 rs1049550* C/T and *BTNL2 rs2076530* G/A SNPs using TaqMan Real-Time PCR Assays. HLA class I/II alleles were typed using PCR sequence-specific primers.

Sixty-six patients experienced disease resolution and 72 developed chronic disease. Comparison of *rs1049550* and *rs2076530* allele frequencies showed no significant differences. Only the HLA *DRB1*03* allele was significantly associated with disease resolution (21.2% vs 4.9% for chronic disease; RR = 0.35; $P < 0.01$ after Bonferroni correction). In the logistic regression models evaluating the association between HLA alleles and chronic sarcoidosis adjusted for *rs1049550* and *rs2076530*, only *DRB1*03* was significantly associated with disease resolution. No significant interactions were found in any of the logistic regression analyses.

In this population of Caucasian patients with sarcoidosis, only *DRB1*03* was associated with disease resolution after 2 years' follow-up, with no significant interactions found for susceptibility gene SNPs *ANXA11 rs1049550* or *BTNL2 rs2076530*.

Key words: sarcoidosis, genetics, HLA, BTNL2, ANXA 11, disease resolution

Introduction

An underlying interaction between a susceptible genetic profile and unknown environmental factors is a widely accepted hypothesis for the pathogenesis of sarcoidosis (1-3). Disease clustering in families, together with different racial and ethnic incidence rates and clinical presentations, lends support to the hypothesis that genetics plays a determining role in disease susceptibility and heterogeneity (1,2). The genes that have been most frequently implicated in sarcoidosis are those encoding antigen presentation and recognition molecules [e.g., human leukocyte antigen (HLA)], cytokines, and receptors (3,4).

Sarcoidosis clinical course is highly variable and ranges from spontaneous resolution to clinical deterioration and onset of lung fibrosis, despite adequate therapeutic intervention (1-3). Differences in outcomes may, to some extent, be related to a genetic network that influences the immunopathogenesis and clinical course of sarcoidosis (4-6).

HLA class I and II alleles were the first consistent genetic associations to be linked to sarcoidosis susceptibility and different clinical presentations and outcomes (7), although their high level of linkage disequilibrium raises the question of whether HLA genes directly determine susceptibility or whether the genetic associations observed are due to other genes in linkage disequilibrium with this region (7-10). This is the case of the butyrophilin-like 2 (*BTNL2)* gene, a member of the immunoglobulin superfamily located at the junction of the HLA class II and class III regions. Considering its amino acid homology to the CD80/CD86 family of costimulatory proteins (3,8,11), this gene appears to have a role in the modulation of costimulatory receptors involved in T-cell responses. Dysfunction of the *BTNL2* protein could thus impair normal T-cell regulation and response to antigens (3,8,11).

Several novel predisposing genes have been identified by genome-wide association studies, and rapid progress in molecular technologies such as systematic and large-scale resequencing will aid the discovery of further risk loci and variants. Hofmann et al. (12), for instance, in a genome-wide search for unknown genetic mutations associated with sarcoidosis susceptibility, evaluated more than 440,000 Single Nucleotide Polymorphisms (SNP) in a population of 499 German patients and 500 controls. The strongest association detected (protective effect on the T allele) was with a SNP (*rs1049550* C/T) in the *ANXA11* gene at chromosome 10q22.3. *ANXA11* is a member of the annexin family of calcium-dependent phospholipid-binding proteins. Although the functional consequences of the *rs1049550* C/T SNP have not been entirely elucidated, *ANXA11* may affect apoptosis pathways, with an imbalance between apoptosis and survival of activated inflammatory cells (12, 13-15).

Of all the genetic polymorphisms analyzed to date, the strongest evidence for an association with sarcoidosis has been found for these particular genetic SNPs (3,11,12).

The aim of the current study was to further investigate associations between disease course in sarcoidosis and HLA class I and II alleles and the susceptibility gene SNPs *ANXA 11 rs1049550* C/T and *BTNL2 rs2076530* G/A, and to explore possible gene-gene interactions in a population of Caucasian patients.

Material and Methods

Patients

All patients included in the study were seen regularly at the interstitial lung diseases outpatient clinic at Centro Hospitalar São João, a tertiary referral center serving patients mostly from the Oporto district and northern Portugal. A biopsy with features compatible with sarcoidosis was obtained in 66% of the patients. All other patients fulfilled the criteria of the consensus statement on sarcoidosis by the European Respiratory Society (ERS)/American Thoracic Society (ATS)/World Association of Sarcoidosis and Other Granulomatous Diseases (WASOG), namely compatible clinical and radiographic features, a bronchoalveolar lavage fluid CD4/CD8 lymphocyte ratio >4.0, and a 2-year observation period to exclude other medical conditions. Löfgren syndrome was defined as the presence of bilateral hilar lymphadenopathy, fever, ankle arthralgia, and erythema nodosum. Thoracic involvement was classified using the Scadding criteria: stage 0, no lung involvement; stage I, bilateral hilar lymphadenopathy without lung involvement; stage II, bilateral hilar lymphadenopathy with lung involvement; stage III, lung involvement only; and stage IV, lung fibrosis. Sarcoidosis resolution was defined as the disappearance of symptoms and normalization of chest radiographs and lung function tests within 2 years of diagnosis. Patients with evidence of disease after 2 years were considered to have chronic sarcoidosis.

Written consent was obtained from all participants, and the study was approved by the ethics committee at our hospital.

Genotyping

Genomic DNA from patients was extracted from peripheral whole blood samples and collected in acid citrate dextrose anticoagulant tubes, using either QIAsymphony or QIAmp DNA kits (Qiagen, Venlo, The Netherlands).

HLA-*A**, -*B**, -*Cw**, -*DRB1**, and -*DQB1** genotyping was performed with PCR-sequence specific oligonucleotides using Luminex technology (Tepnel Lifecodes kits, Stamford, CT, USA) and analyzed with the software Quicktype LifeMatch v2.5 (Tepnel Lifecodes).

Samples were genotyped for the *BTNL2* G→A transition of *rs2076530* using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). Minor groove binding probes were labeled with the fluorescent dyes VIC and FAM. PCR was carried out in a total reaction volume of 12.5 μl with TaqMan SNP Genotyping assay 1x, TaqMan Genotyping Master Mix 1x, and 20 ng of genomic DNA. The amplification

protocol included a denaturation step at 95ºC for 10 min, followed by 40 denaturation cycles at 92ºC for 15 s, and annealing and extension at 60ºC for 1 min. Completed PCRs were read on the ABI PRISM 7000 Sequence Detection Systems and analyzed using SDS 1.1 software for allelic discrimination (Applied Biosystems).

DNA samples were also genotyped for *ANXA 11 rs1049550* C/T (R230C) using a TaqMan SNP genotyping assay (Applied Biosystems) according to the manufacturer's instructions. Minor groove binding probes were labeled with FAM and VIC. PCR was performed in a total reaction volume of 12.5 μl containing TaqMan Genotyping Master Mix 1□, 20 ng of genomic DNA, and the SNP genotyping assay 1□. The amplification protocol began with a denaturation step at 95ºC for 10 min, followed by 40 denaturation cycles at 92ºC for 15 s, and annealing and extension at 60ºC for 1 min. On completion of PCR, the genotype of each sample was automatically determined by measuring allelic-specific fluorescence on the ABI PRISM 7000 Sequence Detection Systems using SDS 1.2 software for allelic discrimination (Applied Biosystems).

Statistical methods

Summary tables (descriptive statistics and/or frequency tables) are provided for all variables in the analysis. Continuous variables are summarized with descriptive statistics (n, mean±SD, range, median, and interquartile range). Frequency counts and percentage of individuals within each category are provided for categorical data. Variable frequencies were determined by direct counting. Differences between groups were evaluated by the X^2 test or the Fisher exact test where appropriate in univariate analyses and by logistic regression in multivariate analyses. HLA, *BTNL2*, and *ANXA* alleles were included in the regression models as independent predictors for sarcoidosis clinical course. Interaction terms were incorporated into the logistic regression models to evaluate multiplicative interaction between alleles. Relative risk (RR) and odds ratios (ORs) with respective 95% confidence intervals (CIs) were also calculated. The Bonferroni method was used to correct for multiple comparisons. *P* values lower than 0.05 were considered statistically significant. SAS Enterprise Guide 4.2 was used for data analysis.

Results

Patients

We studied 138 unrelated Caucasian sarcoidosis patients from the northern region of Portugal; their mean age was 37.2±12.1 years and 78 (56.5%) were women. Thirty patients (21.7%) had Löfgren syndrome. Most patients had pulmonary sarcoidosis: 2 patients (1.4%) had stage 0 disease, 44 (31.8%) had stage I disease, 66 (47.8%) had stage II disease, 10 (7.24%) had stage III disease, and 16 (11.59%) had stage IV disease. Lung function tests revealed an obstructive pattern in 29.5% of the patients, a restrictive pattern in 19.1%, a mixed pattern in 4.3%, and no abnormalities in 46.9%. Evidence of extrathoracic disease was detected in 61 patients (44.2%). The skin (15.9%), liver (7.9%), and eyes (5.79%) were the most commonly affected organs. Sarcoidosis resolved in 66 patients (47.8%) and followed a chronic course in the remaining 72 patients (52.1%) (Table 1).

Genetic SNP associations

On comparing allele frequencies for the *ANXA* rs*1049550* C/T and *BTNL2 rs2076530* G/A SNPs between patients with chronic disease and with resolution, no statistically significant differences were observed (Table 2, 3).

When we compared HLA allele frequencies between the same subgroups of patients in the univariate analysis, the only significant association found after Bonferroni correction for multiple comparisons was for the DRB1*03 allele (4.2% for chronic disease vs 21.2% for resolution; RR = 0.35; $P < 0.01$) (Table 4).

We built logistic regression models to assess the association between HLA alleles and chronic sarcoidosis course adjusting for the *ANXA rs1049550* C/T and *BTNL2 rs2076530* G/A SNPs. In these models, only *DRB1*03* positivity was significantly associated with disease course, with a protective effect observed against chronic disease after correction for multiple comparisons (Table 5). This effect was independent of the *ANXA* and *BTNL2* genotypes. No statistically significant interaction terms were found in any of the logistic regression analyses.

The association between the *DRB1*03* allele and sarcoidosis clinical course remained significant after excluding patients with Löfgren syndrome (who typically have a good prognosis) from the logistic regression model adjusted for the *ANXA* and *BTNL2* SNPs (Table 6).

Discussion

We have investigated a possible interaction effect between three gene regions described as having the strongest association with sarcoidosis. On analyzing HLA class I and II alleles and the *ANXA* and *BTNL2* SNPs, *rs1049550* C/T and *rs2076530* G/A, the only significant association observed was for the HLA class II *DRB1*03* allele, which exerted a protective effect against chronic disease. Moreover, when potential interactions between these SNPs were explored, *DRB1*03* remained the only significant factor for disease course, and no statistically significant interactions were detected between the HLA alleles and SNPs in the logistic regression models.

In a susceptible host, the pathophysiological process in sarcoidosis involves the internalization of antigens; their further processing and presentation by antigen-presenting cells, such as dendritic cells and macrophages; acquisition of T-cell immunity through the trimolecular complex (MHC/peptide/TCR) interaction; generation of specific T-effector cells; activation of macrophages; and induction of granuloma formation (2,6).

Based on current evidence, susceptibility and clinical course in sarcoidosis are determined by different genes, and it is possible that pathophysiological processes are influenced by a complex association of various polymorphisms related to immunological and inflammatory pathways (4,5,16,17). The polymorphisms analyzed in this study have been described in the literature as those with the strongest association with sarcoidosis (4,7,8).

Some features and pathomechanisms in sarcoidosis suggest an autoimmune background, and this is further supported by the occurrence of sarcoidosis in families with other autoimmune diseases (18,19). The MHC region has high linkage disequilibrium, and evolution has ensured that alleles from different loci were kept together in advantageous conserved haplotypes. The most common in Caucasians are HLA-A*01, -C*07, -B*08, and - DRB1*03, but these are also associated with autoimmune diseases (4, 20-23). Recombination events, albeit rare, occur in MHC, and implicated genes may be linked to the telomeric or centromeric part in a broken haplotype, and partial haplotypes of these ancestral haplotypes may hold the susceptibility genes we are looking for. Heat shock proteins (HSP) (5,19,24) and *BTNL2*, one of the non-HLA genes whose polymorphism we studied, are both located in the MHC region.

The HLA/MHC complex, namely class II, is the first and most widely investigated genetic structure related to the immune pathways thought to contribute to sarcoidosis, and in particular to disease susceptibility and clinical course. HLA has been associated with significant ethnic variation, and therefore numerous alleles and haplotypes, especially in class II, have been described as significantly associated with clinical

course in different populations. The alleles HLA-*DRB1*0301* (25-27) and HLA-*DQB1*0201* (28-30) and the haplotype HLA-*DRB1*0401-DPB1*0401* (31) have to date all been associated with favorable prognosis and disease resolution. By contrast, the alleles HLA-*DRB1*12* (9), HLA-*DRB1*1401* (9,27), HLA-*DRB3*0101*(32,33), and HLA-*DQB1*0602* (27-29), and the HLA-*DRB1*1501-DQB1*0602* haplotype (34) have been found to increase the risk for a chronic course. In our population from northern Portugal, only the HLA-*DRB1*03* allele was significantly associated with disease course (after Bonferroni correction for multiple comparisons), with a protective effect seen against chronic disease; this finding is in line with previous reports from other Caucasian populations that *DRB1*03* is a marker of good prognosis (9,25-27). One possible confounding factor is the fact that *DRB1*03* has also been associated with Löfgren syndrome (7,35), but when we removed patients with this subtype of sarcoidosis from the analysis, the protective effect remained significant, lending strength to our results.

Genetic regions other than those of HLA have also been investigated in relation to immune response. Valentonyte et al. (11) were the first to report an association between sarcoidosis susceptibility and the truncating SNP *rs2076530* G/A of the *BTNL2* gene, independently of HLA risk alleles. Additional studies have since been performed in populations from different geographic areas, and while other *BTNL2* SNPs have been explored, and sometimes found to be significantly associated, only *rs2076530* G/A has been consistently linked to sarcoidosis susceptibility (31,36-37). In an earlier study of the same group of patients included in the present study, we found the *BTNL2 rs2076530* G/A SNP to be associated with sarcoidosis susceptibility, but only in patients without Löfgren syndrome (38). Li et al. (40) described an association between *rs2076530* G/A and chronic disease course in a German population, while Coudurier et al. (41) found the same association in three related patients, all with severe forms of sarcoidosis. Although neither of these studies analyzed linkage disequilibrium to *DRB1* alleles, the results do suggest an association between the variant allele and more severe sarcoidosis with progression to fibrosis. In line with the above reports, Wijnen et al. (42), in a Dutch population, reported that the *BTNL2 rs2076530* A allele was more frequent in patients with chronic persistent (progressive) disease than in those with chronic stable disease, independently of the influence of the *DRB1* allele; they specifically found that the *rs2076530* A allele almost doubled the risk of progressive sarcoidosis. More recently, Wennerström et al. (31) also reported an association between the *BTNL2 rs2076530* A allele and persistent disease in a Finnish population, but only when the group with chronic disease was compared with controls, not with patients with disease resolution, as in the previous report by Wijnen et al. (42). In our population we did not find any significant associations between *BTNL2 rs2076530* G/A and sarcoidosis clinical course. Moreover, no differences were found either when the patients were stratified by chronic stable or chronic progressive disease (38).

Since the first report by Hofmann et al. (12) on the significant protective effect of the *ANXA11 rs1049550* C/T SNP in sarcoidosis susceptibility, this finding has been replicated in Czech, American, and Chinese populations (13-15). In our Portuguese population, *ANXA11 rs1049550* C/T was associated with disease susceptibility, but only in patients without Löfgren syndrome (43). While a significant protective effect against sarcoidosis susceptibility has been found for *rs1049550* C/T in different populations, little is known about its association with disease course. Mzarek et al. (13), in a Czech population, and Feng et al. (15), in a Chinese Han population, reported an association between the *ANXA11 rs1049550* T allele and stage I pulmonary sarcoidosis, a mild form of disease that resolves spontaneously in the majority of cases. These observations suggest a possible association with disease resolution, but this has never been reported.

Several questions remain to be solved. Genes linked to sarcoidosis susceptibility do not appear to be necessarily associated with clinical course. We have investigated the genes with the strongest evidence for association to date, but it is possible that the genes most closely linked to sarcoidosis development and course have not yet been described.

In conclusion, the presence of HLA-*DRB1*03* in a Portuguese population was an accurate marker of favorable disease course in patients with sarcoidosis, with no additional influence exerted by the susceptibility SNPs of *BTNL2* and *ANXA11* genes.

Conflicts of interest

The authors have no conflicts of interest to declare

References

1. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am J Respir Crit Care Med* 1999;160(2):736e55.
2. Dominique Valeyre, Antje Prasse, Hilario Nunes, Yurdagul Uzunhan, Pierre-Yves Brillet, Joachim Müller-Quernheim. Sarcoidosis. *Lancet* 2014 Mar 29;383(9923):1155-67.
3. Fischer A., Grunewald J., Spagnolo P., Nebel A., Schreiber S., Müller-Quernheim J. Genetics of Sarcoidosis. *Semin Respir Crit Care Med* 2014; 35(03): 296-306
4. Iannuzzi MC, Rybicki BA. Genetics of sarcoidosis: candidate genes and genome scans. *Proc Am Thorac Soc* 2007; 4: 108-116.
5. Nardi A., Brillet P-Y, Letoumelin P., Girard F., Brauner M., Uzunhan Y., Naccache J-M, Valeyre D, Nunes H. Stage IV sarcoidosis: comparison of survival with the general population and causes of death. *Eur Respir J* 2011; 38: 1368–1373
6. Grunewald J , Spagnolo P, Wahlström J, Eklund A. Immunogenetics of Disease-Causing Inflammation in Sarcoidosis. *Clin Rev Allergy Immunol*. 2015 Mar 20, [Epub ahead of print]
7. Grunewald J, Eklund A. Löfgren's Syndrome, Human Leukocyte Antigen Strongly Influences the Disease Course. *Am J Respir Crit Care Med* Vol 179. pp 307–312, 2009
8. Kishore A, Petrek M. Immunogenetics of sarcoidosis. International Trends in Immunity 2013; 1 (4): 43-53.
9. Sato H, Woodhead FA, Ahmad T, Grutters JC, Spagnolo P, van den Bosch JM, Maier LA, Newman LS, Nagai S, Izumi T, Wells AU, du Bois RM and Welsh KI. Sarcoidosis HLA class II genotyping distinguishes differences of clinical phenotype across ethnic groups. *Hum Mol Genet* 2010;19(20):4100-11.

10. Dubaniewicz A, Dubaniewicz-Wybieralska M, Moszkowska G, Sternau A, Dubaniewicz A. Comparative analysis of DR and DQ alleles occurrence in sarcoidosis and tuberculosis in the same ethnic group: preliminary study. *Sarcoidosis Vasc Diffuse Lung Dis*. 2006 Oct; 23(3): 180-9.
11. Valentonyte R, Hampe J, Huse K et al. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. *Nat Genet* 2005: 37: 357–64.
12. Hofmann S, Franke A, Fischer A et al. Genome-wide association study identifies ANXA11 as a new susceptibility locus for sarcoidosis. *Nat Genet* 2008: 40: 1103–6.
13. Mrazek F, Stahelova A, Kriegova E et al. Functional variant ANXA11 R230C: true marker of protection and candidate disease modifier in sarcoidosis. *Genes Immun* 2011: 12: 490–4.
14. Levin AM, Iannuzzi MC, Montgomery CG et al. Association of ANXA11 genetic variation with sarcoidosis in African Americans and European Americans. *Genes Immun* 2012: 12: 67–77.
15. Feng X, Zang S, Yang Y, Zhao S, Li Y, Gao X, Zhang L. Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population: a case–control study. *BMJ Open* 2014; 4:e004466.
16. Zissel G, Prasse A, Müller-Quernheim J. Immunologic Response of Sarcoidosis. *Semin Respir Crit Care Med* 2010; 31(4): 390-403.
17. Gerke A, Hunninghake G. The Immunology of Sarcoidosis. *Clinics in Chest Medicine* 2008; 29 (3): 379-390.
18. Dubaniewicz A. Microbial and human heat shock proteins as 'danger signals' in sarcoidosis. Hum Immunol. 2013;74:1550-8
19. Dubaniewicz A. Mycobacterium tuberculosis heat shock proteins and autoimmunity in sarcoidosis. Autoimmun Rev. 2010 Apr;9(6):419-24)
20. Hedfors E and Lindstrom F. HLA-B8/DR3 in sarcoidosis. Correlation to acute onset disease with arthritis. *Tissue Antigens* 1983;22(3):200-3
21. Lio D, Candore G, Romano GC, D'Anna C, Gervasi F, Di Lorenzo G, Modica MA, Potestio M, Caruso C. Modification of cytokine patterns in subjects bearing the HLA-B8,DR3 phenotype: implications for autoimmunity. *Cytokines Cell Mol Ther* 1997;3:217–24.
22. Candore G, Lio D, Colonna Romano G, Caruso C. Pathogenesis of autoimmune diseases associated with 8.1 ancestral haplotype: effect of multiple gene interactions. Autoimmun Rev. 2002;1(1-2):29-35.
23. Giuseppina Candore , Maria Assunta Modica , Domenico Lio Giuseppina Colonna-Romano , Florinda Listì, Maria Paola Grimaldi, Mariangela Russo, Giovanni Triolo , Antonia Accardo-Palumbo , Maria Clara Cuccia, Calogero Caruso; Pathogenesis of autoimmune diseases associated with 8.1 ancestral haplotype: a genetically determined defect of C4 influences immunological parameters of healthy carriers of the haplotype; Biomedicine & Pharmacotherapy 57 (2003) 274–277.
24. Ishiara M, Shigeaky O. Genetic influences on sarcoidosis. Eye 1997; 11, 155-161.
25. Wennerstrom A, Pietinalho A, Vauhkonen H, Lahtela L, Palikhe A, Hedman J, Purokivi M, Varkki E, Seppanen M, Lokki ML, Selroos O. HLA-DRB1 allele frequencies and C4 copy number variation in Finnish sarcoidosis patients and associations with disease prognosis. *Hum Immunol* 2012;73(1):93-100.

399 26. Gardner J, Kennedy HG, Hamblin A and Jones E. HLA associations in sarcoidosis:
400 a study of two ethnic groups. *Thorax* 1984;39(1):19-22.
401 27. Berlin M, Fogdell-Hahn A, Olerup O, Eklund A and Grunewald J. HLA-DR
402 predicts the prognosis in Scandinavian patients with pulmonary sarcoidosis. *Am J*
403 *Respir Crit Care Med* 1997;156(5):1601-5.
404 28. Iannuzzi MC, Maliarik MJ, Poisson LM and Rybicki BA. Sarcoidosis susceptibility
405 and resistance HLA-DQB1 alleles in African Americans. *Am J Respir Crit Care*
406 *Med* 2003;167(9):1225-31.
407 29. Sato H, Grutters JC, Pantelidis P, Mizzon AN, Ahmad T, Van Houte AJ, Lammers
408 JW, Van Den Bosch JM, Welsh KI and Du Bois RM. HLA-DQB1*0201: a marker
409 for good prognosis in British and Dutch patients with sarcoidosis. *Am J Respir Cell*
410 *Mol Biol* **2002**;27(4):406-12.
411 30. van den Berg-Loonen EM, Voorter CEM and Drent M. Strong association of severe
412 pulmonary sarcoidosis with HLA DQB1*0602. *Hum Immunol* 2004;65:S34-S34.
413 31. Wennerstrom A, Pietinalho A, Lasota J, Salli K, Surakka I, Seppanen M, Selroos O
414 and Lokki ML. Major histocompatibility complex class II and BTNL2 associations
415 in sarcoidosis. *Eur Respir J* 2013;42(2):550-3.
416 32. Bogunia-Kubik K, Tomeczko J, Suchnicki K and Lange A. HLA-DRB1*03,
417 DRB1*11 or DRB1*12 and their respective DRB3 specificities in clinical variants
418 of sarcoidosis. *Tissue Antigens* 2001;57(1):87-90.
419 33. Ishihara M, Ishida T, Mizuki N, Inoko H, Ando H and Ohno S. Clinical features of
420 sarcoidosis in relation to HLA distribution and HLA-DRB3 genotyping by PCR-
421 RFLP. *Br J Ophthalmol* 1995;79(4):322-5.
422 34. Voorter CE, Drent M and van den Berg-Loonen EM. Severe pulmonary sarcoidosis
423 is strongly associated with the haplotype HLA-DQB1*0602-DRB1*150101. *Hum*
424 *Immunol* 2005;66(7):826-35.
425 35. Morais A, Alves H, Lima B, Delgado L, Goncalves R, Tafulo S. HLA class I and II
426 and TNF-alpha gene polymorphisms in sarcoidosis patients. *Rev Port Pneumol*
427 2008;14:727-46.
428 36. Rybicki BA, Walewski JL, Maliarik MJ, Kian H, Iannuzzi MC. The ACCESS
429 Research Group. The BTNL2 gene and sarcoidosis susceptibility in African
430 Americans and Whites. *Am J Hum Genet* 2005;77:491-9.
431 37. Milman N, Svendsen CB, Nielsen FC, Hansen TO. The BTNL2 A allele variant is
432 frequent in Danish patients with sarcoidosis. *Clin RespirJ* 2011;5:105-11.
433 38. Morais A, Lima B, Peixoto MJ, Alves H, Marques A, Delgado L. BTNL2 gene
434 polymorphism associations with susceptibility and phenotype expression in
435 sarcoidosis. *Respir Med* 2012;106(12):1771-7
436 39. Gaillot-Drevon M, Calender A, Blay JY, Valeyre D, Israel-Biet D, Roy P, Pacheco
437 Y. Lack of correlation of BTNL2 polymorphism and cancer risk in sarcoidosis.
438 *Sarcoidosis Vasc Diffuse Lung Dis*. 2014 Jul 8; 31(2):136-41.
439 40. Li Y, Pabst S, Lokhande S, Grohe C and Wollnik B. Extended genetic analysis of
440 BTNL2 in sarcoidosis. *Tissue Antigens* 2009;73(1):59-61.
441 41. Coudurier M, Freymond N, Aissaoui S, Calender A, Pacheco Y and Devouassoux
442 G. Homozygous variant rs2076530 of BTNL2 and familial sarcoidosis. *Sarcoidosis*
443 *Vasc Diffuse Lung Dis* 2009;26(2):162-6.
444 42. Wijnen PA, Voorter CE, Nelemans PJ, Verschakelen JA, Bekers O, Drent M.
445 Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and
446 progression? *Hum Immunol* 2011;72:342-7.

43. Morais A, Lima B, Peixoto M, et al. Annexin A11 gene polymorphism (R230C variant) and sarcoidosis in a Portuguese population. *Tissue Antigens* 2013; 82:186–91.

Diagnostic value of CD103 expression in bronchoalveolar lymphocytes in sarcoidosis

Patrícia Caetano Mota, António Morais, Carmo Palmares, Marília Beltrão, Natália Melo, Ana Cristina Santos, Luís Delgado

Respiratory Medicine (2012) 106, 1014–1020

Available online at www.sciencedirect.com

Diagnostic value of CD103 expression in bronchoalveolar lymphocytes in sarcoidosis

Patrícia Caetano Mota [a], António Morais [a,b,*], Carmo Palmares [c], Marília Beltrão [c], Natália Melo [a], Ana Cristina Santos [d,e], Luís Delgado [c]

[a] *Pulmonology Department, Centro Hospitalar de São João, EPE, 4200-319 Porto, Portugal*
[b] *Faculdade de Medicina da Universidade do Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal*
[c] *Immunology Department, Faculdade de Medicina da Universidade do Porto, 4200-319 Porto, Portugal*
[d] *Clinical Epidemiology, Predictive Medicine and Public Health Department, Faculdade de Medicina da Universidade do Porto, 4200-319 Porto, Portugal*
[e] *Instituto de Saúde Pública da Universidade do Porto, 4200-319 Porto, Portugal*

Received 17 November 2011; accepted 27 March 2012
Available online 24 April 2012

KEYWORDS
Sarcoidosis;
Bronchoalveolar lavage fluid;
$CD103^+$

Summary
Background: Pulmonary sarcoidosis is frequently characterized by a $CD4^+/CD8^+$ ratio $\geq$3.5 in bronchoalveolar lavage fluid (BALF), although up to 40% of the cases present a normal or even decreased ratio, pointing out its variability and limitation as a diagnostic marker for sarcoidosis. Lung lymphocytes within the bronchial epithelium, the alveolar walls, and BALF express the integrin CD103. Our aim was to compare the expression of CD103 in BALF T-lymphocytes between sarcoidosis and other interstitial lung diseases (ILD) and to evaluate its relevance as a BALF diagnostic marker for sarcoidosis.
Methods: A total of 86 patients with ILD (mean age $\pm$ standard deviation, 42.6 $\pm$ 16.6 years; 60.5% female), who underwent BALF as part of their initial diagnostic work-up, were enrolled into 2 groups: sarcoidosis (n = 41) and other ILD (n = 45). Area under the receiver operating characteristic (ROC) curve (AUC) was used to describe the performance of CD103 for sarcoidosis diagnosis.
Results: Sarcoidosis patients presented a significantly reduced CD103 expression in BALF T-lymphocytes, more pronounced in the $CD4^+$ subset. The BALF $CD103^+CD4^+/CD4^+$ ratio for a cutoff point of 0.45 was associated with a better diagnostic performance for sarcoidosis (AUC: 0.86 [95% confidence interval (95% CI): 0.78—0.94]; sensitivity: 81%; specificity: 78%), even for those with a $CD4^+/CD8^+$ ratio <3.5 (AUC: 0.79 [95% CI: 0.64—0.93]; sensitivity: 75%; specificity: 78%).

* Corresponding author. Faculdade de Medicina da Universidade do Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal. Tel.: +351 22 5512100; fax: +351 22 5512214.
E-mail address: amorais3@hotmail.com (A. Morais).

0954-6111/$ - see front matter
doi:10.1016/j.rmed.2012.03.020

Conclusions: Assessment of CD103 expression in BALF $CD4^+$ T-lymphocytes may be a reliable tool for sarcoidosis diagnosis, independently of $CD4^+/CD8^+$ ratio, pointing out the relevance of evaluating the $CD103^+CD4^+/CD4^+$ ratio in the ILD diagnostic work-up.

Introduction

Sarcoidosis is the most common interstitial lung disease (ILD) in western world, with an estimated prevalence of 10–20 per 100,000 people.[1,2] It is a multisystem inflammatory disorder of unknown origin that commonly affects young adults. It is characterized by the accumulation of macrophages and $CD4^+$ T-lymphocytes in involved organs, with non-caseating granuloma formation, being the lungs, lymph nodes and skin the most frequently affected.[3,4]

The diagnosis of pulmonary sarcoidosis encloses a correct clinical setting, typical chest radiographic or high-resolution computed tomography (HRCT) features and a biopsy showing non-caseating granulomas.[3,5–7] Bronchoalveolar lavage fluid (BALF) is considered as a standard procedure in the diagnostic work-up of patients with ILD. In sarcoidosis the value of the BALF $CD4^+/CD8^+$ ratio to differentiate it from other ILD has been examined by several authors, which conclude that in patients with a typical clinical picture an elevated BALF $CD4^+/CD8^+$ ratio may support the diagnosis of sarcoidosis and obviate the need for confirmation by additional biopsy.[8–10] Those studies demonstrated that a $CD4^+/CD8^+$ ratio greater than 3.5 shows a high specificity of 93–96% for sarcoidosis, although the sensitivity is low, approximately 52–59%.[8,9,11,12] On the other hand, other studies[13] question the clinical usefulness of the BALF $CD4^+/CD8^+$ ratio, based on the observation that this ratio is highly variable. These authors found that only 42% of 86 patients with biopsy-proven sarcoidosis had a BALF ratio greater than 4.0, and that 12% had even an inverted ratio below 1.0, reflecting a predominance of $CD8^+$ T-lymphocytes.

Consequently, there has been a great interest in the investigation of other cellular markers with a reliable diagnostic accuracy for sarcoidosis. In this context, the expression of CD103 (integrin $\alpha_E\beta_7$) in BALF $CD4^+$ lymphocytes has been shown to be a promising candidate.[14]

Terminally differentiated mucosal intraepithelial T-lymphocytes express the integrin $\alpha_E\beta_7$, an adhesion molecule and homing receptor that binds to E-cadherin, a molecule vital for the adhesion and retention of epithelial cells.[15] In the lung, lymphocytes within the bronchial epithelium, the alveolar walls, and BALF express the integrin CD103,[16,17] although with a variable magnitude of expression.[18] In fact, a great variation of the CD103 expression in BALF $CD4^+$ and $CD8^+$ T-cells has been reported in ILD, suggesting a possible role of these subpopulations in the pathogenesis and differential diagnosis of some of these disorders.[19] Kolopp-Sarda et al. found that sarcoidosis was remarkably characterized by the lack of CD103 expression in the predominant $CD4^+$ subset.[14] The peripheral origin of $CD4^+$ BALF lymphocytes in sarcoidosis seems to be responsible for its lower expression of CD103.[14] These observations may be relevant for the diagnosis of sarcoidosis, namely through the analysis of $CD103^+CD4^+$ cell expression, along with the BALF $CD4^+/CD8^+$ and even BALF/peripheral blood ratios.[14,20]

Thus, we aimed to compare the expression of CD103 in BALF T-lymphocytes between patients with diagnosis of sarcoidosis and other ILD, and to evaluate its relevance as a BALF diagnostic marker for sarcoidosis.

Methods

Study population

A total of 86 patients with a confirmed ILD diagnosis (mean age ± standard deviation, 42.6 ± 16.6 years; 60.5% female), who underwent BALF as part of their initial diagnostic work-up, were enrolled in the study. Patients were divided into 2 groups for comparison effects, namely sarcoidosis ($n = 41$) and other ILD ($n = 45$).

Sarcoidosis diagnosis was based on a multimodality approach that combined clinical, radiological, and histological evaluation showing non-caseating granulomas, according to the American Thoracic Society/European Respiratory Society/World Association for Sarcoidosis and Other Granulomatous Disorders (ATS/ERS/WASOG) statement.[5] This diagnosis was also considered in those with clinical and radiological features typical of sarcoidosis associated with a BALF $CD4^+/CD8^+$ lymphocyte ratio $\geq$3.5. Chest radiographic staging was performed for all sarcoidosis patients according to Scadding criteria: stage 0 – normal ($n = 1$), stage I – mediastinal and bilateral hilar lymphadenopathy without lung involvement ($n = 14$), stage II – lymphadenopathy and lung involvement ($n = 21$), stage III – only lung involvement ($n = 0$), stage IV – lung fibrosis ($n = 5$).[5,21] A group of 22 patients had a diagnosis of hypersensitivity pneumonitis (HP), according to Schuyler et al.'s criteria.[22] The diagnosis of idiopathic interstitial pneumonia was based on surgical lung biopsy features or in accordance to the ATS/ERS International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias: idiopathic pulmonary fibrosis (IPF) ($n = 4$); non-specific interstitial pneumonia (NSIP) ($n = 3$); cryptogenic organizing pneumonia (COP) ($n = 1$).[23] Connective tissue disease-associated lung disorders were diagnosed according to international proposed criteria associated with HRCT scan and BALF features: systemic lupus erythematosus (SLE) ($n = 3$); rheumathoid arthritis ($n = 3$); scleroderma ($n = 2$).[24–26] Drug-induced lung disease (3 by rapamycin and 1 by capecitabine) and silicosis ($n = 3$) diagnosis were based on a compatible exposure history, radiological and BALF features.[27,28]

This study was approved by Ethics Committee of Centro Hospitalar de São João.

Bronchoalveolar lavage and flow cytometry

BAL was performed according to the ERS recommendations,[29] by flexible bronchoscopy at the time of diagnosis. Briefly, four aliquots of 50 mL sterile isotonic saline solution (37 °C) were instilled under fiberoptic bronchoscopy in the middle lobe and gently aspirated with a syringe (after each instillation). Recovered BALF was pooled (discarding the first aliquot), gauze filtered and the total cell numbers (Neubauer chamber) determined. Cell differentials were obtained by counting 500 cells on cytospin preparations stained with Wright-Giemsa. For phenotypic analysis, cells were centrifuged at 250 G for 10 min, washed twice, resuspended, and labeled with the following combination of monoclonal antibodies: anti-CD103-FITC, anti-CD8-Pe, anti-CD45-PercP-Cy.5 and anti-CD4-APC (Becton Dickinson Immunocytometry Systems, BDIS, San José, CA, USA). CD3, CD19, and CD56 expressions were also assessed in a parallel sample. Cells were acquired on a FACScan Flow Cytometer, using CellQuestPro Software (Beckton and Dickinson, BD Biosciences, San José, USA). Lymphocyte gating was based on forward scatter (FSC) versus side scatter (SSC). Additional gating was based on SSC versus CD45, CD4 and CD8 populations. BALF $CD4^+$, $CD8^+$, $CD103^+CD4^+$ and $CD103^+CD8^+$ T-lymphocytes were analyzed.

Statistical analysis

Data were described as mean and standard deviation or as median and interquartile range (IQR) for quantitative variables, and as counts and proportions. For comparison of quantitative variables the Student's *t*-test and the Mann—Whitney test were used according to variables distribution.

Sensitivity and specificity were calculated for a set of cutoff points of the various calculated forms of CD103 expression in BALF $CD4^+$ and $CD8^+$ T-lymphocytes.

The area under the receiver operating characteristic (ROC) curve (AUC), which plots sensitivity against 1 − specificity, was used to describe the diagnostic performance of those variables. Positive and negative predictive values (PPV and NPV) were also estimated.

Statistical analyses were performed using the Statistical Package for Social Sciences for Windows version 18 (SPSS; Chicago, IL, USA). Statistical significance was denoted by a *p*-value lower than 0.05 for all tests performed.

Results

Expression of CD103 in BALF T-lymphocytes

A significantly higher $CD4^+/CD8^+$ ratio in BALF was found for sarcoidosis patients compared to patients with other ILD (Table 1). The comparative analysis of the CD103 expression in the $CD4^+$ and $CD8^+$ T-lymphocyte subpopulations ($CD103^+CD4^+$ cells/μL; $CD103^+CD4^+$ %; $CD103^+CD4^+/CD4^+$; $CD103^+CD8^+$ cells/μL; $CD103^+CD8^+$ %; $CD103^+CD8^+/CD8^+$; $CD103^+CD4^+/CD103^+CD8^+$) between both groups (sarcoidosis versus other ILD) is represented in Table 1. As expected, in the sarcoidosis group, the absolute counts and percentages were significantly lower compared to the other group. Moreover, CD103 expression in the $CD4^+$ T-lymphocyte subset was lower than CD103 in $CD8^+$ T-cells.

As compared to other ILD, BALF $CD103^+CD4^+/CD4^+$ ratio, reflecting the relative number of $CD4^+$ T-lymphocytes that express CD103 within the total $CD4^+$ subpopulation, was also lower − 0.20 (IQR 0.25) versus 1.00 (IQR 1.50), $p < 0.001$ − in sarcoidosis patients (Table 1). This ratio was significantly higher in more advanced radiographic stages (stages ≤1: 0.10 (IQR 0.10) versus stages ≥2: 0.20 (IQR 0.43); $p = 0.044$).

Diagnostic criteria of BALF CD103 expression (sensitivity, specificity, cutoff levels)

Sets of cutoff points were established for the different values of CD103 expression in BALF T-lymphocytes, calculating their respective sensitivity and specificity values for sarcoidosis diagnosis (data available as supplementary material − Annexes 1 and 2). In accordance, and for the same cutoff ≤0.25, BALF $CD103^+CD4^+/CD4^+$ and

Table 1 Comparative analysis of T-lymphocyte subpopulations and $CD103^+$ expression between patients with sarcoidosis and other interstitial lung disease (ILD).

	Sarcoidosis ($n = 41$)	Other ILD ($n = 45$)	*p*-value
Lymphocytes (%)	34.8 (30.5)	53.0 (43.7)	0.014
$CD4^+$ (%)	79.4 (15.7)	38.0 (35.5)	<0.001
$CD8^+$ (%)	14.8 (11.7)	46.4 (33.1)	<0.001
$CD4^+/CD8^+$	5.7 (5.1)	0.9 (1.3)	<0.001
$CD103^+CD4^+$ (cells/μL)	5411 (19,179)	11,185 (43,215)	0.024
$CD103^+CD4^+$ (%)	11.9 (14.5)	32.6 (44.2)	<0.001
$CD103^+CD4^+/CD4^+$	0.20 (0.25)	1.00 (1.50)	<0.001
$CD103^+CD8^+$ (cells/μL)	2441 (6923.5)	42,646 (147357.5)	<0.001
$CD103^+CD8^+$ (%)	27.3 (46.6)	64.0 (55.6)	<0.001
$CD103^+CD8^+/CD8^+$	2.00 (2.35)	1.2 (1.45)	0.018
$CD103^+CD4^+/CD103^+CD8^+$	0.50 (0.60)	0.60 (0.50)	0.199

Quantitative variables are expressed as median and interquartile range (IQR).

Table 2 Sensitivity (Sn) and specificity (Sp) of the BALF $CD103^+CD4^+/CD4^+$ ratio for sarcoidosis diagnosis in the study population (left column), and in a subgroup of sarcoidosis with a BALF CD4/CD8 <3.5 (right column) all with histological confirmation, comparatively with the other ILD.

All sarcoidosis patients ($n = 41$)			Sarcoidosis with BALF $CD4^+/CD8^+$ <3.5 ($n = 12$)		
$CD103^+CD4^+/CD4^+$	Sn (%)	Sp (%)	$CD103^+CD4^+/CD4^+$	Sn (%)	Sp (%)
0.05	12	100	0.15	25	93
0.15	42	93	0.25	42	91
0.25	63	91	0.35	58	80
0.35	76	80	0.45	75	78
0.45	81	78	0.55	75	71
0.55	83	71	0.65	83	64
0.65	88	64	0.75	83	60
0.75	88	60	0.85	83	56
0.85	90	56	0.95	83	53
0.95	93	53	1.05	83	49

Sn, sensitivity; Sp, Specificity.

$CD103^+CD4^+/CD103^+CD8^+$ ratios were highly specific for sarcoidosis (specificity >90% for both), but with a low effect in sensitivity values (sensitivity for $CD103^+CD4^+/CD4^+$ <63% and for $CD103^+CD4^+/CD103^+$ $CD8^+$ <20%) (Table 2 and Annex 1).

ROC curves were performed in order to better evaluate the diagnostic value of CD103 BALF expression in sarcoidosis and to determine the cutoff values with the best sensibility and specificity relationship. Among the aforementioned variables, the $CD103^+CD4^+/CD4^+$ ratio, for a cutoff point of 0.45, was associated with a better diagnostic performance (sensitivity: 81%; specificity: 78%; PPV: 77%; NPV: 81%) (Table 3), as shown by ROC curves (Fig. 1a) and respective AUC (AUC: 0.86 [95% CI: 0.78–0.94]). In comparison, $CD103^+CD4^+/CD103^+CD8^+$ ratio, for the same cutoff value (0.45), despite a similar specificity, had a low sensitivity for sarcoidosis and consequently a lower AUC (sensitivity: 44%; specificity: 73%; PPV: 60%; NPV: 59%; AUC: 0.58 [95% CI: 0.46–0.70]) (Table 3 and Fig. 1c).

CD103 expression in sarcoidosis patients with BALF $CD4^+/CD8^+$ ratio < 3.5

Among the 41 sarcoidosis patients included, 12 (29.3%) presented a BALF $CD4^+/CD8^+$ <3.5 but with histological evaluation showing non-caseating granulomas. Similar statistical analysis was performed for this subgroup. In comparison with the others parameters, the $CD103^+CD4^+/CD4^+$ ratio (for the same cutoff point, 0.45, determined for the overall sarcoidosis group) revealed the best diagnostic performance, as shown by AUC magnitude (sensitivity: 75%; specificity: 78%; PPV: 47%; NPV: 92%; AUC: 0.79 [95% CI: 0.64–0.93]) (Table 4 and Fig. 1b).

Discussion

Sarcoidosis is characterized by an alveolar $CD4^+$ lymphocytosis, the effector cells involved in the T-helper 1 immune response, and consequently a high BALF $CD4^+/CD8^+$ ratio.[3,4,8–10] Although with a high specificity, an elevated $CD4^+/CD8^+$ ratio does not fully discriminate sarcoidosis from other ILD.[13] $CD4^+$ lymphocytosis may also be observed in other types of lung diseases,[30] and the sensitivity for sarcoidosis of an elevated $CD4^+/CD8^+$ ratio is seldom more than 50% in the studies available in literature.[8,9,11] Additionally, difficulties also rise in those cases of sarcoidosis with a normal or inverted $CD4^+/CD8^+$ ratio. Thus, other BALF cellular markers that may help in sarcoidosis differential diagnosis have been searched.

Table 3 Cutoff values, sensitivity (Sn), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) for different bronchoalveolar lavage fluid (BALF) criteria evaluated for sarcoidosis diagnosis.

Criteria	Selected cutoff	Sn (%)	Sp (%)	PPV (%)	NPV (%)	AUC (CI 95%)
$CD103^+CD4^+$ (cells/μL)	7286	59	69	63	65	0.64 (0.53–0.76)
$CD103^+CD4^+$ (%)	17.85	71	80	76	75	0.75 (0.66–0.87)
$CD103^+CD4^+/CD4^+$	0.45	81	78	77	81	0.86 (0.78–0.94)
$CD103^+CD8^+$ (cells/μL)	19,954	98	62	70	97	0.84 (0.76–0.93)
$CD103^+CD8^+$ (%)	46.9	71	69	67	72	0.72 (0.61–0.83)
$CD103^+CD8^+/CD8^+$	1.35	71	60	62	69	0.65 (0.53–0.77)
$CD103^+CD4^+/CD103^+CD8^+$	0.45	44	73	60	59	0.58 (0.46–0.70)

Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under curve; CI, confidence interval.

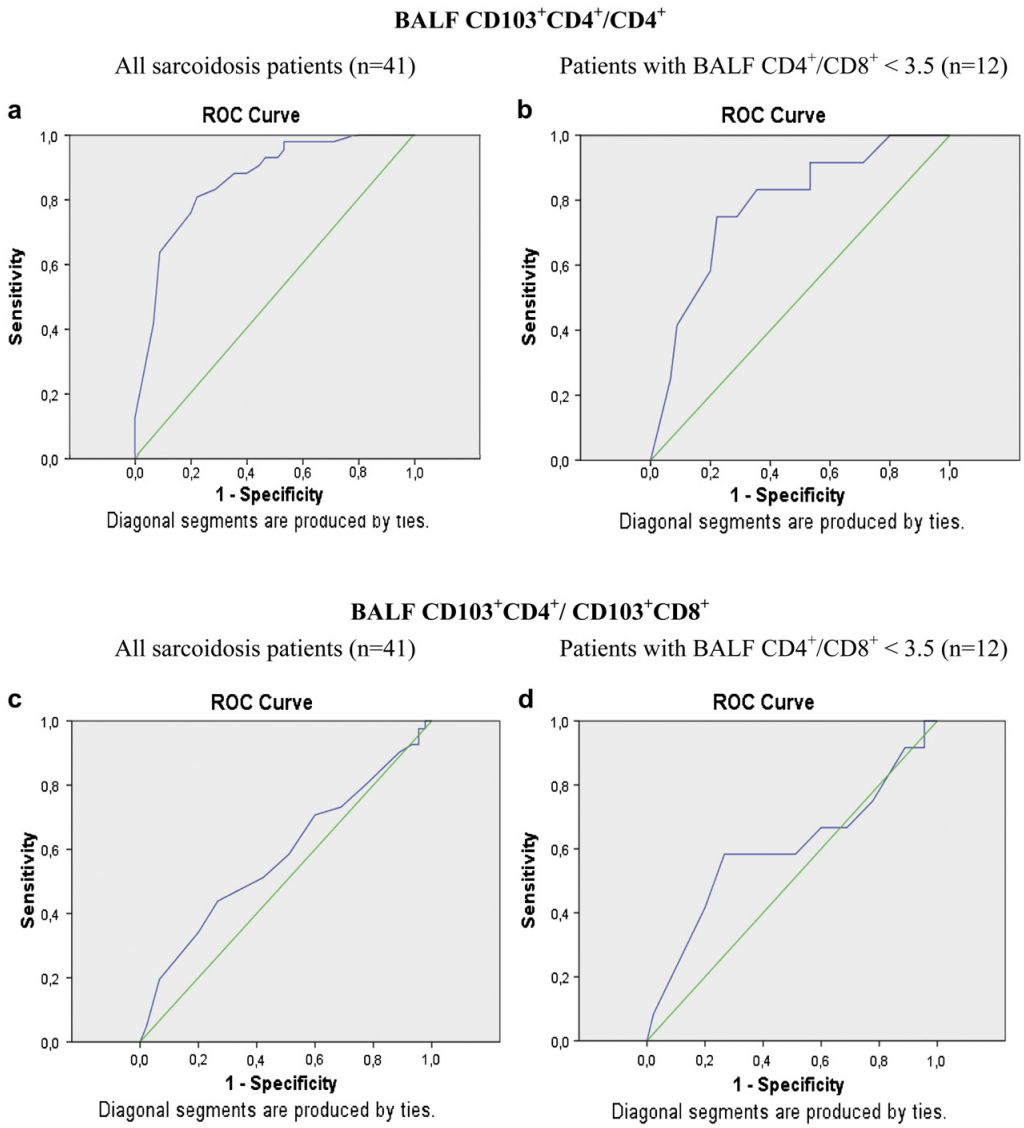


Figure 1 Receiver operating characteristic (ROC) curves of the bronchoalveolar lavage fluid (BALF) $CD103^{+}CD4^{+}/CD4^{+}$ and $CD103^{+}CD4^{+}/CD103^{+}CD8^{+}$ ratios in sarcoidosis patients independently of $CD4^{+}/CD8^{+}$ ratio (a, c) and for those with a $CD4^{+}/CD8^{+}$ ratio <3.5 (b, d), respectively.

In this context, the integrin CD103, expressed on intra-epithelial lymphocytes in mucosal areas, such as bronchi, has been studied in the diagnostic work-up of sarcoidosis. It has been shown that the relative amount of CD103-expressing T-cells in BALF differs in patients with ILD. This variation is predominantly seen in the $CD4^{+}$ T-cell population, as most of the $CD8^{+}$ T-cells express this integrin independently of the type of disease.[18] This was also shown in our study, as a low expression of $CD103^{+}$ was seen on $CD4^{+}$ rather than on $CD8^{+}$ BALF T-lymphocytes. Subsequently, a significantly lower BALF $CD103^{+}CD4^{+}/CD4^{+}$ ratio was also found in this group of patients. As other have suggested, this is corroborative with the concept that, for instance, lymphocytosis in HP results from the local expansion of mucosal lymphocytes, while in sarcoidosis, is the result of lymphocytes of non-mucosal origin. In fact, the relative absence of CD103 on $CD4^{+}$ BALF T-lymphocytes is consistent with a peripheral origin of these cells, favoring the hypothesis of redistribution from the peripheral blood and compartmentalization into the lung.[14,20,31,32] In fact, in

Table 4 Cutoff values, sensitivity (Sn), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) for different bronchoalveolar lavage fluid (BALF) criteria evaluated for sarcoidosis diagnosis for patients with BALF $CD4^+/CD8^+$ ratio <3.5 ($n = 12$; 29.3%).

Criteria	Selected cutoff	Sn (%)	Sp (%)	PPV (%)	NPV (%)	AUC (CI 95%)
$CD103^+CD4^+$ (cells/μL)	2951	50	84	46	86	0.69 (0.53—0.85)
$CD103^+CD4^+$ (%)	18.85	75	76	45	92	0.74 (0.57—0.91)
$CD103^+CD4^+/CD4^+$	0.45	75	78	47	92	0.79 (0.64—0.93)
$CD103^+CD8^+$ (cells/μL)	16,856	100	64	43	100	0.77 (0.65—0.89)
$CD103^+CD8^+$ (%)	27.65	50	82	43	86	0.64 (0.46—0.82)
$CD103^+CD8^+/CD8^+$	1.65	50	69	30	84	0.48 (0.29—0.68)
$CD103^+CD4^+/CD103^+CD8^+$	0.45	58	73	37	87	0.60 (0.39—0.80)

Sn, sensitivity; Sp, specificity; PPV, positive predictive value (PPV); NPV, negative predictive value; AUC, area under curve; CI, confidence interval.

our study BALF CD103 expression adequately differentiated sarcoidosis from the HP group, that showed a clearly higher BALF $CD103^+CD4^+/CD4^+$ ratio (data not shown).

Our results support the relevance to evaluate CD103 expression in BALF $CD4^+$ T-lymphocytes in sarcoidosis. Independently of the $CD4^+/CD8^+$ ratio, the BALF $CD103^+CD4^+/CD4^+$ ratio for a cutoff of 0.25 presented a high specificity (91%) for sarcoidosis diagnosis (Table 2). In order to characterize the diagnostic performance of this ratio, and to calculate the best relationship between sensitivity and specificity, we determined the cutoff value associated with the highest AUC value for this ratio, and for other CD103 expression values. In fact, the $CD103^+CD4^+/CD4^+$ ratio, for the cutoff 0.45, presented the highest AUC value, with a sensitivity and specificity of 81% and 78%, respectively. Taking into account these results, one can speculate that the $CD103^+CD4^+/CD4^+$ ratio ranging from 0.25 to 0.45 can be a useful tool for sarcoidosis diagnosis (Annex 1).

The potential diagnostic role of CD103 in sarcoidosis has been previously demonstrated by some authors. We cannot perform a direct comparison between our results and those found by these groups, as methodologies used differed. Kolopp-Sarda et al. have found that the combined use of the $CD4^+/CD8^+$ (≥ 2.5) and the $CD103^+/CD4^+$ ratio (<0.31) seems to be a promising new tool for sarcoidosis diagnosis, with sensitivity around 96%.[14] On the other hand, Heron et al. have already demonstrated, in a total of 119 patients with alveolar lymphocytosis (56 with pulmonary sarcoidosis), that the combined use of the $CD103^+CD4^+/CD4^+$ ratio (cutoff <0.2) with the $CD4^+/CD8^+$ (cutoff >3.0), or with the relative alveolitis $CD4^+/CD8^+$ BAL/periphery blood ratio (cutoff >2.0), provides a specific tool for discriminating sarcoidosis from other ILD (sensitivity: 66%; specificity: 89%; PPV: 82%; NPV: 74%).[20] As opposed to the aforementioned study, this last work, performed by the Dutch BAL working party, has some advantages, such as the inclusion of sarcoidosis patients with a $CD4^+/CD8^+$ <2.5, revealing the additional usefulness of CD103 in those patients without an apparent high $CD4^+$ alveolitis. Moreover, the application of the $CD103^+CD4^+/CD4^+$ ratio rather than the $CD103^+/CD4^+$ ratio, prevents the misleading effect of $CD8^+$ lymphocytes, that co-express CD103 in most lung diseases. Similarly, we did not evaluate CD103 global expression and we included patients with a great variability of BALF $CD4^+/CD8^+$ ratio. Among our 41 sarcoidosis patients, 12 presented a $CD4^+/CD8^+$ <3.5 (including 4 patients with an inverted $CD4^+/CD8^+$ <1). In this subgroup of patients, the $CD103^+CD4^+/CD4^+$ ratio for the same cutoff point (0.45), as it was established for the total sample, revealed a similar diagnostic performance, with an expected higher negative predictive value (92%). This emphasizes the potential diagnostic role of this ratio also in sarcoidosis patients with a normal or inverted $CD4^+/CD8^+$ ratio, obviating the need of more invasive diagnostic tools in order to obtain a histological confirmation. Our sample also differed in the diagnostic criteria applied for sarcoidosis, as we included not only patients with biopsy-proven diagnosis (the unique criteria applied in the former studies), but also, patients with a high $CD4^+/CD8^+$ ratio in the presence of a clinical/radiological picture typical of this diagnosis without histological confirmation. Consequently, we cannot compare the diagnostic values of $CD4^+/CD8^+$ ratio and CD103 expression for sarcoidosis, as independent or combined markers, as others have shown. Although this is a potential limitation of our study, we currently should not subject patients with a typical clinical, radiological and BALF features of sarcoidosis to invasive tests, in order to obtain a histological confirmation.

Additionally, the small number of patients with BALF $CD4^+/CD8^+$ ratio <3.5 could be considered a potential limitation of this study. Nevertheless, our results are in line with those previously described by Heron et al.,[20] concerning the diagnostic value of CD103 expression even in sarcoidosis patients with BALF CD4/CD8 ratio <3.5.

As described in the literature, higher values of the BALF $CD103^+CD4^+/CD4^+$ ratio were found in more advanced sarcoidosis radiographic stages.[19,20] $CD103^+$ cells are involved in fibrogenic inflammation, as shown by an analysis of single nucleotide polymorphisms spanning ITGAE, the gene encoding the αE (CD103) unit, suggesting that the genotypic analysis of this integrin may also be helpful in assessing sarcoidosis risk and prognosis.[17,33]

In conclusion, assessment of CD103 expression in BALF $CD4^+$ lymphocytes may be a reliable tool for sarcoidosis diagnosis, independently of $CD4^+/CD8^+$ ratio, pointing out the relevance of evaluating this marker on the ILD diagnostic work-up. In our study, through an extensive analysis of sensitivity and specificity of various forms of its expression we were able to redefine a BALF $CD103^+CD4^+/CD4^+$ lymphocyte ratio ≤ 0.45 as a good diagnostic marker for sarcoidosis, even in cases with $CD4^+/CD8^+$ ratio <3.5.

Conflict of interest

The authors have no conflict of interest.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rmed.2012.03.020.

References

1. Hillerdal G, Nöu E, Osterman K, Schmekel B. Sarcoidosis: epidemiology and prognosis. A 15-year European study. *Am Rev Respir Dis* 1984;**130**:29—32.
2. Henke CE, Henke G, Elveback LR, Beard CM, Ballard DJ, Kurland LT. The epidemiology of sarcoidosis in Rochester, Minnesota: a population-based study of incidence and survival. *Am J Epidemiol* 1986;**123**:840—5.
3. Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. *N Engl J Med* 2007;**357**:2153—65.
4. Nureki S, Miyazaki E, Ando M, Ueno T, Fukami T, Kumamoto T, et al. Circulating levels of both Th1 and Th2 chemokines are elevated in patients with sarcoidosis. *Respir Med* 2008;**102**:239—47.
5. Costabel U, Hunninghake GW. ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders. *Eur Respir J* 1999;**14**:735—7.
6. Baughman RP, Culver DA, Judson MA. A concise review of pulmonary sarcoidosis. *Am J Respir Crit Care Med* 2011;**183**:573—81.
7. Bradley B, Branley HM, Egan JJ, Greaves MS, Hansell DM, Harrison NK, et al. British Thoracic Society Interstitial Lung Disease Guideline GroupBritish Thoracic Society Standards of Care CommitteeThoracic Society of AustraliaNew Zealand Thoracic SocietyIrish Thoracic Society. Interstitial lung disease guideline: the British Thoracic Society in collaboration with the Thoracic Society of Australia and New Zealand and the Irish Thoracic Society. *Thorax* 2008;**63**(Suppl. 5):v1—58.
8. Costabel U, Zaiss AW, Guzman J. Sensitivity and specificity of BAL findings in sarcoidosis. *Sarcoidosis* 1992;**9**(Suppl. 1):211—4.
9. Winterbauer RH, Lammert J, Selland M, Wu R, Corley D, Springmeyer SC. Bronchoalveolar lavage cell populations in the diagnosis of sarcoidosis. *Chest* 1993;**104**:352—61.
10. Reichenberger F, Kleiber B, Baschiera B, Bubendorf L, Brutsche M, Dalquen P, et al. Bronchoalveolar lavage quality influences the T4/T8 ratio in sarcoidosis. *Respir Med* 2007;**101**:2025—30.
11. Thomeer M, Demedts M. Predictive value of CD4/CD8 ratio in bronchoalveolar lavage in the diagnosis of sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1997;**14**(Suppl. 1):36 (Abstract).
12. Costabel U. CD4/CD8 ratios in bronchoalveolar lavage fluid: of value for diagnosing sarcoidosis? *Eur Respir J* 1997;**10**:2699—700.
13. Kantrow SP, Meyer KC, Kidd P, Raghu G. The CD4/CD8 ratio in BAL fluid is highly variable in sarcoidosis. *Eur Respir J* 1997;**10**:2716—21.
14. Kolopp-Sarda MN, Kohler C, De March AK, Béné MC, Faure G. Discriminative immunophenotype of bronchoalveolar lavage CD4 lymphocytes in sarcoidosis. *Lab Invest* 2000;**80**:1065—9.
15. Kilshaw PJ. Alpha E beta 7. *Mol Pathol* 1999;**52**:203—7.
16. Erle DJ, Pabst R. Intraepithelial lymphocytes in the lung: a neglected lymphocyte population. *Am J Respir Cell Mol Biol* 2000;**22**:398—400.
17. Braun RK, Foerster M, Grahmann PR, Haefner D, Workalemahu G, Kroegel C. Phenotypic and molecular characterization of CD103+CD4+ T cells in bronchoalveolar lavage from patients with interstitial lung diseases. *Cytometry B Clin Cytom* 2003;**54**:19—27.
18. Rihs S, Walker C, Virchow Jr JC, Boer C, Kroegel C, Giri SN, et al. Differential expression of alpha E beta 7 integrins on bronchoalveolar lavage T lymphocyte subsets: regulation by alpha 4 beta 1-integrin crosslinking and TGF-beta. *Am J Respir Cell Mol Biol* 1996;**15**:600—10.
19. Lohmeyer J, Friedrich J, Grimminger F, Maus U, Tenter R, Morr H, et al. Expression of mucosa-related integrin alphaE-beta7 on alveolar T cells in interstitial lung diseases. *Clin Exp Immunol* 1999;**116**:340—6.
20. Heron M, Slieker WA, Zanen P, van Lochem EG, Hooijkaas H, van den Bosch JM, et al. Evaluation of CD103 as a cellular marker for the diagnosis of pulmonary sarcoidosis. *Clin Immunol* 2008;**126**:338—44.
21. Kirks DR, McCormick VD, Greenspan RH. Pulmonary sarcoidosis. Roentgenologic analysis of 150 patients. *Am J Roentgenol Radium Ther Nucl Med* 1973;**117**:777—86.
22. Schuyler M, Cormier Y. The diagnosis of hypersensitivity pneumonitis. *Chest* 1997;**111**:534—6.
23. American Thoracic Society/European Respiratory Society. International multidisciplinary consensus classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2002;**165**:277—304.
24. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;**40**:1725.
25. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315—24.
26. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. *Arthritis Rheum* 1980;**23**:581—90.
27. http://www.pneumotox.com/.
28. Adverse effects of crystalline silica exposure. American Thoracic Society Committee of the Scientific Assembly on Environmental and Occupational Health. *Am J Respir Crit Care Med* 1997;**155**:761—8.
29. Technical recommendations and guidelines for bronchoalveolar lavage (BAL). Report of the European Society of Pneumology Task Group. *Eur Respir J* 1989;**2**:561—85.
30. Agostini C, Chilosi M, Zambello R, Trentin L, Semenzato G. Pulmonary immune cells in health and disease: lymphocytes. *Eur Respir J* 1993;**6**:1378—401.
31. Semenzato G. Immunology of interstitial lung diseases: cellular events taking place in the lung of sarcoidosis, hypersensitivity pneumonitis and HIV infection. *Eur Respir J* 1991;**4**:94—102.
32. Chailleux E, Bignon JD, Peyrat MA, Godard A, Soulillou JP. Lymphocyte subsets, phytohaemagglutinin responsiveness of blood lymphocytes, and interleukin 2 production in sarcoidosis. *Thorax* 1985;**40**:768—73.
33. Heron M, Grutters JC, Van Moorsel CH, Ruven HJ, Kazemier KM, Claessen AM, et al. Effect of variation in ITGAE on risk of sarcoidosis, CD103 expression, and chest radiography. *Clin Immunol* 2009;**133**:117—25.

Integrin aEb7 (CD103) expression in bronchoalveolar lymphocytes of patients with hypersensitivity pneumonitis

Mariana Couto, Carmo Palmares, Marília Beltrão, Sofia Neves, Patrícia Mota, António Morais, Luís Delgado

International Archives Occupational Environment Health (2015) 88:167–173

Int Arch Occup Environ Health (2015) 88:167–173
DOI 10.1007/s00420-014-0947-4

ORIGINAL ARTICLE

Integrin $\alpha_E\beta_7$ (CD103) expression in bronchoalveolar lymphocytes of patients with hypersensitivity pneumonitis

Mariana Couto · Carmo Palmares · Marília Beltrão · Sofia Neves · Patrícia Mota · António Morais · Luís Delgado

Received: 21 October 2012 / Accepted: 18 May 2014 / Published online: 30 May 2014

Abstract
Purpose CD4+/CD8+ ratio in bronchoalveolar lavage fluid (BALF) often retrieves contradictory findings when used for diagnosis of sarcoidosis and hypersensitivity pneumonitis (HP), so CD103+ has been investigated as a possible differential marker. We aimed to compare CD103+ expression in BALF T-lymphocytes between patients with HP, sarcoidosis and other interstitial lung diseases (ILD).
Methods An observational study carried out over a 2-year period included consecutive patients with suspected ILD who underwent BALF as part of their initial diagnostic work-up; CD103+ expression on BALF T-lymphocytes was evaluated. After a final diagnosis established according to international criteria, three patient subgroups—HP, ILD (which included idiopathic interstitial pneumonia and connective tissue disease-associated lung disorders) and sarcoidosis—were considered for further analysis.
Results A total of 77 subjects were enrolled, 20 with HP, 16 with other ILD and 41 with sarcoidosis. A significantly higher number of CD4+ CD103+ and CD8+ CD103+ lymphocytes were found in HP patients. Among patients with sarcoidosis, 12 (29.3 %) presented a BALF CD4+/CD8+ <3.5, all of them with histological confirmation. Compared to these patients, also statistically significant higher CD4+ CD103+ counts in HP patients were observed ($p = 0.007$). Among HP patients, although bird fanciers ($n = 14$) presented higher percentages of both CD4+ CD103+ and CD8+ CD103+ T-lymphocytes than those with work-related HP ($n = 5$), the differences did not reach statistical significance.
Conclusions Patients with HP present significantly higher counts of CD103+ T-lymphocytes in BALF, both in the CD4+ and CD8+ subsets, when compared to sarcoidosis, even with sarcoidosis subgroup presenting a BALF CD4+/CD8+ <3.5. The expression of CD103 may help in the interpretation of BALF data in these diffuse granulomatous lung disorders.

Keywords Hypersensitivity pneumonitis · Bronchoalveolar lavage · CD103 · Sarcoidosis

M. Couto (✉) · C. Palmares · M. Beltrão · A. Morais · L. Delgado
Serviço e Laboratório de Imunologia, Faculdade de Medicina, Universidade do Porto, Alameda Prof Hernâni Monteiro, 4200-319 Porto, Portugal
e-mail: marianafercouto@gmail.com

M. Couto · L. Delgado
Serviço de Imunoalergologia, Centro Hospitalar São João, E.P.E., Porto, Portugal

S. Neves
Serviço de Pneumologia, Centro Hospitalar Vila Nova de Gaia e Espinho, E.P.E., Porto, Portugal

P. Mota · A. Morais
Serviço de Pneumologia, Centro Hospitalar São João, E.P.E., Porto, Portugal

Introduction

Interstitial lung diseases are a heterogeneous group of more than 150 entities that involve the lung's parenchyma—the alveoli, the alveolar epithelium, the capillary endothelium and the spaces between these structures—as well as the perivascular and lymphatic tissues (Behr 2012). Among ILDs, those with a predominant granulomatous reaction in the interstitial or vascular areas are especially relevant by their prevalence and pathological features (Fauci et al. 2008). Sarcoidosis and hypersensitivity pneumonitis, being the most common granulomatous lung disorders, differ in many other aspects.

Sarcoidosis is a multisystem disease, with heterogeneous clinical presentations, characterized by the accumulation of CD4+ T helper lymphocytes in affected tissues. This translates into the high CD4+/CD8+ ratio usually found in patients' bronchoalveolar lavage fluid (BALF) (Costabel et al. 1992; Winterbauer et al. 1993; Iannuzzi et al. 2007; Reichenberger et al. 2007). A BALF CD4+/CD8+ ratio greater than 3.5 shows a high diagnostic specificity (93 to 96 %) for lung sarcoidosis. Therefore, in patients with a suggestive clinical/radiological picture of lung sarcoidosis, an elevated BALF CD4+/CD8+ ratio may support the diagnosis and obviate the need for confirmatory lung biopsy (Costabel et al. 2010). However, the sensitivity is low (53 to 59 %) (Costabel et al. 1988; Winterbauer et al. 1993; Thomeer and Demedts 1997; Korosec et al. 2010), with only 55 % of patients showing an increased CD4+/CD8+ ratio at the time of diagnosis, and the ratio may even decrease below 1.0 in 15 % of individuals with sarcoidosis (Costabel et al. 2010).

Hypersensitivity pneumonitis (HP), although only restricted to the lung, has also a typical lymphocytic inflammation in peripheral airways and surrounding interstitial tissue, with the formation of granulomas (Patel et al. 2001). These appear to be the result of a classic delayed (T lymphocyte-mediated) hypersensitivity reaction to repeated antigen inhalation (Kline and Hunninghake 2008). However, BALF lymphocytosis in HP is mostly due to the CD8+ subset, resulting in the typical low CD4+/CD8+ ratio in BALF. However, also in HP, contradictory findings of decreased, normal or increased CD4+/CD8+ BALF ratios have been shown (Barrera et al. 2008; Ye et al. 2009). The ratio is usually higher in chronic or subacute HP and in those with bird fanciers disease (Morais et al. 2004; Barrera et al. 2008), and therefore, a high BALF CD4+/CD8+ ratio does not rule out the diagnosis of HP.

Previous studies of CD103 expression in BALF T-lymphocytes of patients with sarcoidosis (Heron et al. 2008; Mota et al. 2012) showed a characteristic low expression (Kolopp-Sarda et al. 2000), consistent with a peripheral origin of these cells (Cerf-Bensussan et al. 1987). Little is known about the CD103 expression in HP, but as an antigen-driven hypersensitivity disorder associated with the exposure and inhalation of occupational antigens, it is possible that the lymphocytic alveolitis seen in HP results from the local expansion of mucosal/intraepithelial lymphocytes (CD103+), in contrast with recruitment from a systemic pool, as proposed in sarcoidosis (Semenzato 1991; Heron et al. 2008; Mota et al. 2012). Therefore, in this study, we aimed to compare the CD103 expression in BALF T-lymphocytes in patients with these granulomatous lung disorders.

Patients and methods

An observational study was carried out over a 2-year period. Consecutive patients with suspected ILD who underwent BALF as part of their initial diagnostic work-up were enrolled, and CD103+ expression on BALF T-lymphocytes was evaluated. After a final diagnosis established according to international criteria, three patient subgroups were considered for further analysis: HP, sarcoidosis or other ILD (which included idiopathic interstitial pneumonia and connective tissue disease-associated lung disorders). Demographic and clinical data were further extracted from clinical files.

Sarcoidosis diagnosis was based on a multimodality approach that combined clinical, radiological and histological evaluation showing noncaseating granulomas, according to the American Thoracic Society/European Respiratory Society/World Association for Sarcoidosis and Other Granulomatous Disorders (ATS/ERS/WASOG) statement (Costabel and Hunninghake 1999).

The diagnosis of idiopathic interstitial pneumonia was based on surgical lung biopsy features or in accordance with the ATS/ERS International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias: idiopathic pulmonary fibrosis (IPF) ($n = 4$); nonspecific interstitial pneumonia (NSIP) ($n = 3$); cryptogenic organizing pneumonia (COP) ($n = 1$) (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias 2002).

Connective tissue disease-associated lung disorders were diagnosed according to EULAR proposed criteria associated with HRCT scan and BALF features: systemic lupus erythematosus (SLE) ($n = 3$); rheumatoid arthritis ($n = 3$); and scleroderma ($n = 2$) (Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee 1980; Arnett et al. 1988; Hochberg 1997).

Diagnosis of hypersensitivity pneumonitis was established according to Schuyler et al. criteria (Schuyler and Cormier 1997). High-resolution computed tomography (HRCT) was classified as presenting ground glass opacities, mosaic pattern and fibrosis (Selman et al. 2010).

Antigenic exposure among patients with HP was ascertained by detailed questionnaire of patient's workplace and hobby exposures, questioning about the most common antigens in our country (mold, organic dust and bird exposures) and the duration of contact in years.

BAL was performed according to the ERS recommendations (European Society of Pneumology Task Group 1989) by flexible bronchoscopy at the time of diagnosis. Cells were acquired on a FACScan Flow Cytometer, using CellQuestPro Software (Beckton and Dickinson, BD

Biosciences, San José, USA). Lymphocyte gating was based on forward scatter (FSC) versus side scatter. Additional gating was based on SSC versus CD45, CD4 and CD8 populations. BALF CD4+, CD8+, CD4+CD103+ and CD8+CD103+ T-lymphocytes were analyzed.

Statistical Package for Social Sciences for Windows version 20.0 (SPSS; Chicago, IL, USA) was used, considering a significance level of 0.05. Data for quantitative variables were described as median and interquartile range (except for age, in which minimum and maximum values are presented). For comparison of quantitative variables, the Mann–Whitney or the Kruskal–wallis test were used. Categorical variables were compared using Qui-square or Fisher's exact test.

The study was conducted in accordance with the Declaration of Helsinki for Medical Research Involving Human Subjects and was approved by Ethics Committee of Centro Hospitalar São João.

Results

A total of 77 subjects were enrolled, among which 20 were diagnosed with HP, 16 with other ILDs and 41 with sarcoidosis; in the remaining, no disease confirmation was achieved. Among patients diagnosed with HP, the majority were males (14 patients—70 %), never smokers (n = 14–70 %) and the median age was 46 years (min 11–max 75); sarcoidosis patients were mostly females (30 patients—73 %), never smokers (n = 35–85 %) with median age of 33 years (min 17–max 70). Patients with other ILD were almost all females (n = 14–88 %), never smokers (n = 14–88 %) and with median age of 62 years (min 19–max 77) (Table 1).

BALF characteristics, T-lymphocytes and subpopulations among patients with HP, other ILDs and sarcoidosis are presented in Table 2. A significantly higher number of both BALF CD4+CD103+ and CD8+CD103+ lymphocytes were found in HP patients compared to sarcoidosis ($p < 0.001$ and $p = 0.002$, respectively) and with other ILDs ($p = 0.001$ and $p = 0.01$, respectively) (Fig. 1).

Among patients with sarcoidosis, 12 (29.3 %) presented a BALF CD4+/CD8+ <3.5 but with histological evaluation confirming noncaseating granulomas. Compared to this subgroup, we also found statistically significant higher CD4+CD103+ relative counts in BALF of HP patients ($p = 0.007$); for CD8+CD103+, although higher relative counts were also seen, the difference did not reach a statistical significance ($p = 0.087$).

Among HP patients, the mean antigenic exposure time was 20 years (10–35): 14 were recreationally exposed to birds and five had work-related HP, such as mold exposure in suberosis (Morais et al. 2004)—1 was excluded from this analysis as he presented both concomitant exposures. Although bird fanciers presented higher percentages of both CD4+CD103+ and CD8+CD103+ T-lymphocytes, the differences did not reach statistical significance (Table 3). Only one patient with HP presented fibrosis in the HRCT scan.

Table 1 Patients with hypersensitivity pneumonitis (HP) compared to sarcoidosis and interstitial pneumonitis (IP)

	HP (n = 20)	Sarcoidosis (n = 41)	IP (n = 16)
Males	14 (70)	11 (27)	2 (13)
Age, median (IQR)	46 (11–75)	33 (17–70)	62 (19–77)
Smoking habits			
Never smoker	14 (70)	35 (85)	14 (88)
Current smoker	3 (15)	3 (7.5)	1 (0.6)
Past smoker	3 (15)	3 (7.5)	1 (0.6)
HRCT[a]			
Ground glass/mosaic pattern	19 (95)	–	–
Mosaic pattern, fibrosis and honeycombing	1 (5)	–	–
Scadding stage 0	–	1 (2.5)	–
Scadding stage I	–	18 (45)	–
Scadding stage II	–	18 (45)	–
Scadding stage III	–	1 (2.5)	–
Scadding stage IV	–	2 (5)	–
Nonspecific interstitial pneumonia pattern	–	–	9 (57)
Usual interstitial pneumonia pattern	–	–	5 (31)
Organizing pneumonia pattern	–	–	2 (12)
Respiratory functional pattern[b]			
Normal	5 (42)	12 (50)	2 (29)
Obstructive ventilatory pattern	1 (8)	3 (12.5)	1 (14)
Restrictive ventilatory pattern	6 (50)	6 (25)	4 (57)
Mixed ventilatory pattern	0	3 (12.5)	0

Data presented as n (%), except for age

HRCT high-resolution computerized tomography scan

[a] Missing data for 1 patient with sarcoidosis

[b] Missing data for 8 patients with HP, 17 patients with sarcoidosis and 9 with interstitial pneumonitis

Discussion

Our study showed that patients with HP present statistically significant higher counts of CD103+ T-lymphocytes in BALF, both in the CD4+ and CD8+ subsets, when compared to other granulomatous diffuse lung disorder such as

Table 2 Bronchoalveolar lavage fluid characteristics, T cells subpopulations and CD103+ expression in patients with hypersensitivity pneumonitis (HP) compared to sarcoidosis and interstitial pneumonitis (IP)

	HP ($n = 20$)	Sarcoidosis ($n = 41$)	IP ($n = 16$)	p
Fluid recovery (mL)	104.5 (81.3–129.5)	120.0 (110.0–133.5)	114.5 (93.5–131.8)	0.169
Total cell counts				
%	4.4 (2.8–10.1)	1.4 (1.0–3.3)	2.1 (1.0–4.1)	<0.001
Cells/μl	500,000 (320,000–940,000)	210,000 (100,000–410,000)	140,000 (100,000–320,000)	<0.001
Macrophages				
%	21.6 (12.5–26.9)	56.8 (41.6–70.0)	56.0 (44.6–72.9)	<0.001
Neutrophils				
%	5.6 (2.4–8.9)	1.6 (0.7–2.6)	4.8 (1.3–7.4)	<0.001
Eosinophils				
%	0.8 (0.2–2.9)	0.4 (0.1–0.8)	1.2 (0.4–3.8)	0.049
Mastocytes				
%	0.4 (0–0.8)	0.0 (0.0–0.2)	0.0 (0.0–0.2)	0.005
Multinuclear cells				
%	0.4 (0–0.9)	2.0 (1.5–3.4)	1.9 (1.4–3.2)	<0.001
T-lymphocytes				
%	70.8 (64.7–77.5)	34.8 (24.6–55.1)	27.6 (18.0–48.7)	<0.001
Cells/μl	478,220 (189,760–648,720)	32,880 (17,960–106,890)	72,120 (23,210–105,350)	<0.001
CD4 +				
%	29.9 (13.4–43.2)	79.4 (68.6–84.3)	56.7 (35.9–70.8)	<0.001
Cells/μl	98,614 (35,975–250,751)	26,118 (10,650–83,959)	33,788 (14,163–48,382)	0.002
CD8 +				
%	59.4 (44.1–82.6)	14.8 (10.4–22.1)	38.4 (25.9–57.1)	<0.001
Cells/μl	209,473 (103,711–476,154)	7,717 (1,533–20,535)	18,882 (6,800–59,264)	<0.001
CD4+/CD8+ ratio	0.6 (0.2–1.1)	5.7 (3.0–8.1)	1.5 (0.7–2.7)	<0.001
CD4+ CD103+				
%	38.0 (25.7–65.7)	11.9 (7.3–21.8)	24.0 (18.1–42.6)	0.003
Cells/μl	56,460 (6,741–98,563)	3,050 (1,059–15,639)	8,055 (4,148–14,512)	<0.001
CD8+ CD103+				
%	64.5 (41.2–89.4)	27.3 (13.1–59.7)	51.9 (25.7–79.2)	0.002
Cells/μl	117,970 (57,452–265,295)	2,269 (507–7,171)	4,702 (2,656–25,658)	<0.001

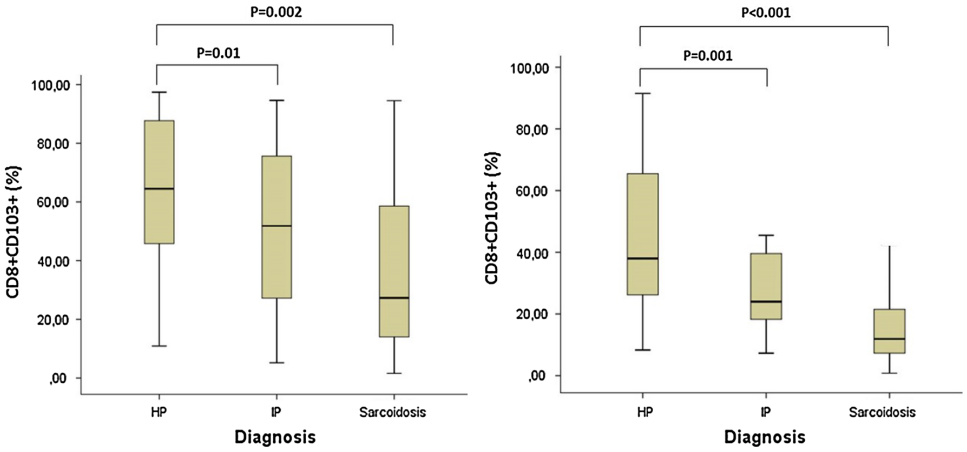


Fig. 1 Expression of CD103+ on bronchoalveolar lavage fluid T-lymphocytes in patients with hypersensitivity pneumonitis (HP), sarcoidosis and interstitial pneumonitis (IP)

Table 3 T-lymphocyte subpopulations and CD103+ expression in bronchoalveolar lavage fluid of patients with hypersensitivity pneumonitis and different occupational exposures (bird fanciers or professionally exposed to other antigens)

	Bird fanciers ($n = 14$)	Suberosis/other work-related antigens ($n = 5$)	p
T-lymphocytes			
Cells/μl	550,880 (93,760–1,737,600)	259,920 (177,760–658,240)	0.41
%	70.8 (53.0–84.6)	73.2 (40.4–83.0)	1.00
CD4+			
Cells/μl	116,022 (6,731–507,504)	48,351 (13,280–220,510)	0.17
%	34.6 (5.7–61.6)	27.2 (3.2–45.9)	0.46
CD8+			
Cells/μl	241,966 (37,692–1,391,818)	216,773 (104,530–398,815)	0.78
%	48.9 (3.9–91.5)	67.2 (47.6–96.1)	0.17
CD4+/CD8+ ratio	0.7 (0.1–6.0)	0.3 (0–1.0)	0.25
CD4+ CD103+			
Cells/μl	74,409 (1,339–334,445)	11,185 (4,013–76,517)	0.08
%	59.8 (12.7–91.5)	34.7 (8.3–43.3)	0.27
CD8+ CD103+			
Cells/μl	142,544 (35,091–643,956)	52,683 (37,628–285,689)	0.23
%	75.6 (19.0–97.4)	50.4 (10.9–83.8)	0.15

sarcoidosis, as well as when compared to the group of idiopathic interstitial pneumonias and connective tissue disease-associated lung disorders. These findings corroborate the hypothesis that BALF T lymphocytosis in HP alveolitis results from the local expansion of mucosal/intraepithelial lymphocytes (CD103+), rather than recruitment from a systemic pool as occurs in sarcoidosis (Semenzato 1991; Heron et al. 2008). Furthermore, comparatively with a sarcoidosis subgroup presenting a BALF CD4+/CD8+ <3.5, also a significant percentage of CD4+CD103+ in BALF was seen in HP patients. Among patients with HP presenting different antigenic exposures, no significant differences were observed in CD103 expression.

The main limitations of this study are the small sample size and its observational nature, which does not allow establishing causal relationships.

A few studies have addressed the possible role of CD103 as a cellular marker in diffuse lung diseases, and it has been demonstrated that the relative amount of CD103-expressing T-lymphocytes in the BALF differs in patients with ILD, depending on the type of disease, and predominantly in the CD4+ T cell population. Patients with idiopathic pulmonary fibrosis (IPF) and hypersensitivity pneumonitis were shown to have a significantly higher proportion of CD4+ T-lymphocytes expressing CD103 compared to patients with sarcoidosis (Lohmeyer et al. 1999; Kolopp-Sarda et al. 2000). Our study confirms the previous data and also extends the same findings to the CD8+ T-lymphocyte subpopulation.

Moreover, this study looked from the HP perspective and evaluated the usefulness of this marker in the context of the differential diagnosis of its BALF profile. Given that HP is a granulomatous disease like sarcoidosis, sometimes with a similar presentation, and that CD4+/CD8+ ratio in BALF of patients with sarcoidosis and HP can be highly variable (Kantrow et al. 1997; Barrera et al. 2008; Ye et al. 2009; Costabel et al. 2010), new markers that can improve differential diagnosis between sarcoidosis and HP are desirable. Our data show that assessment of CD103 expression on CD4+ BALF T-lymphocytes is of additional value in the differential diagnostic work-up of patients with suspected granulomatous diffuse pulmonary diseases. Furthermore, other features of BALF in HP patients can impose additional difficulties to diagnosis. For instance, the cellular profile also relates to the antigenic exposure: CD8+ suppressor lymphocytes predominate in BALF of patients recently exposed to antigens and gradually decrease in number with cessation of exposure (Costabel et al. 1984), with a parallel increase in CD4+ T-lymphocytes (Drent et al. 1993). In addition, neutrophilia is characteristic during the acute post-exposure phase (Navarro et al. 2006), particularly during the first 48 h (Ohtani et al. 2000). The measurement of T-lymphocytes expressing the integrin CD103 can be valuable in the context of variable CD4/CD8 ratios.

It could be speculated that the different expression of CD103+ underlies the immunopathological basis for the clinical differences observed among these two conditions. For instance, the fact that patients with sarcoidosis present lower CD103+ T-lymphocytes in BALF may relate to a peripheral origin of these cells (Cerf-Bensussan et al. 1987), in accordance with the multisystemic nature of the disease, while in HP, the bronchoalveolar local expansion of mucosal/intraepithelial lymphocytes (CD103+) could support the restriction to the respiratory surface. On the

other hand, it is known that CD4+CD103+ lymphocytes are mainly found within the T cell memory compartment and have regulatory capacity, suppressing T cell proliferation (Lehmann et al. 2002). Moreover, after activation, this CD4+CD103+ subpopulation may express a unique Th2 cytokine pattern with emphasis on IL-13 (Lehmann et al. 2002), which has pro-fibrotic properties and mediates induction of TGF-β1-dependent tissue fibrosis (Fichtner-Feigl et al. 2006). So, it is possible that the predominance of CD103+ BALF lymphocytes seen in HP reflects a repairing phase (e.g., suppression and/or fibrosis) of the granulomatous and interstitial inflammation. Nevertheless, in our sample, only one patient had HCRT changes compatible with fibrosis and therefore further studies with longer follow-up will be determinant to clarify this question.

Among all types of HP, bird fanciers lung is one of the most frequently diagnosed in our region, together with suberosis due to cork working industries in our area (Morais et al. 2004; Winck et al. 2004a, b). Despite the intensive antigen exposure that occurs in these two forms of HP, only a small part of the subjects develops clinically significant HP disease. Its development has been pointed out to depend on the type, intensity and duration of exposure to the inciting agent, as well as the genetic susceptibility of the host (Melo et al. 2006), the site of interaction within the respiratory system and the resulting level of immune response dysregulation over time (Patel et al. 2001). The intensity of exposure among subjects that contact with antigens within recreational exposure to birds differs from the occupational inhalation that cork industry workers experience during their five work days job (Morais et al. 2004). It would be conceivable that different CD103+ expression in lung lymphocytes could reflect these different levels and type of exposure. However, in our preliminary observations, we could not find a significant difference between the two groups, although a higher expression of CD103+ was seen among bird fanciers. Future studies with larger samples are needed to investigate the CD103+ BALF expression among HP patients with different exposures and clinical presentations.

Conclusion

Our study showed that patients with hypersensitivity pneumonitis present statistically significant higher numbers of CD103+ T-lymphocytes in BALF, both in the CD4+ and CD8+ subsets, which corroborates prior observations and the hypothesis that BALF T lymphocytic alveolitis results from the local expansion of mucosal/intraepithelial lymphocytes (CD103+). Furthermore, the expression of CD103 can help in the interpretation of BALF data in diffuse lung disorders.

Conflict of interest The authors declare that they have no conflict of interest.

References

American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus (2002) Classification of the idiopathic interstitial pneumonias. Am J Respir Crit Care Med 165:277–304

Arnett FC, Edworthy SM et al (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31(3):315–324

Barrera L, Mendoza F et al (2008) Functional diversity of T-cell subpopulations in subacute and chronic hypersensitivity pneumonitis. Am J Respir Crit Care Med 177(1):44–55

Behr J (2012) Approach to the diagnosis of interstitial lung disease. Clin Chest Med 33:1–10

Cerf-Bensussan N, Jarry A et al (1987) A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. Eur J Immunol 17:1279–1285

Costabel U, Bonella F et al (2010) Diagnostic modalities in sarcoidosis: BAL, EBUS, and PET. Semin Respir Crit Care Med 31(4):404–408

Costabel U, Bross K et al (1984) T-lymphocytosis in bronchoalveolar lavage fluid of hypersensitivity pneumonitis. Changes in profile of T-cell subsets during the course of disease. Chest 85:514–522

Costabel U, Hunninghake GW (1999) ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders. Eur Respir J 14(4):735–737

Costabel U, Zaiss A et al (1992) Sensitivity and specificity of BAL findings in sarcoidosis. Sarcoidosis 9(Suppl 1):211–214

Costabel U, Zaiss A et al (1988) Value of bronchoalveolar lavage lymphocyte subpopulations for the diagnosis of sarcoidosis. In: Grassi C, Rizzato G, Pozzi E (eds) Sarcoidosis and other granulomatous disorders. Elsevier, Amsterdam, pp 429–432

Drent M, Van Velzen-Blad H et al (1993) Bronchoalveolar lavage in extrinsic allergic alveolitis: effect of time elapsed since antigen exposure. Eur Respir J 6:1276–1281

European Society of Pneumology Task Group (1989) Technical recommendations and guidelines for bronchoalveolar lavage (BAL). Report of the European Society of Pneumology Task Group. Eur Respir J 2:561–585

Fauci A, Braunward E et al (2008) Interstitial lung diseases. Harrison's principles of internal medicine. McGraw-Hill, NY

Fichtner-Feigl S, Strober W et al (2006) IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med 12(1):99–106

Heron M, Slieker W et al (2008) Evaluation of CD103 as a cellular marker for the diagnosis of pulmonary sarcoidosis. Clin Immunol 126:338–344

Hochberg MC (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40(9):1725

Iannuzzi M, Rybicki B et al (2007) Sarcoidosis. N Engl J Med 357:2153–2165

Kantrow S, Meyer K et al (1997) The CD4/CD8 ratio in BAL fluid is highly variable in sarcoidosis. Eur Respir J 12:21

Kline J, Hunninghake G (2008) Hypersensitivity pneumonitis and pulmonary infiltrates with eosinophilia. Harrison's principles of internal medicine. McGraw-Hill, NY

Kolopp-Sarda M, Kohler C et al (2000) Discriminative immunophenotype of bronchoalveolar lavage CD4 lymphocytes in sarcoidosis. Lab Invest 80:1065–1069

Korosec P, Rijavec M et al (2010) Deficiency of pulmonary Valpha24 Vbeta11 natural killer T cells in corticosteroid-naive sarcoidosis patients. Respir Med 104(4):571–577

Lehmann J, Huehn J et al (2002) Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25 + as well as CD25- regulatory T cells. Proc Natl Acad Sci USA 99(20):13031–13036

Lohmeyer J, Friedrich J et al (1999) Expression of mucosa-related integrin alphaEbeta7 on alveolar T cells in interstitial lung diseases. Clin Exp Immunol 116:340–346

Melo N, Morais A et al (2006) Major histocompatibility complex and cytokines polymorphisms in hypersensitivity pneumonitis. Eur Resp J 28:829s

Morais A, Winck JC et al (2004) Suberosis and bird fancier's disease: a comparative study of radiological, functional and bronchoalveolar lavage profiles. J Investig Allergol Clin Immunol 14(1):26–33

Mota PC, Morais A et al (2012) Diagnostic value of CD103 expression in bronchoalveolar lymphocytes in sarcoidosis. Respir Med 106(7):1014–1020

Navarro C, Mejia M et al (2006) Hypersensitivity pneumonitis: a broader perspective. Treat Respir Med 5:167–179

Ohtani Y, Kojima K et al (2000) Inhalation provocation tests in chronic bird fancier's lung. Chest 118:1382–1389

Patel A, Ryu J et al (2001) Hypersensitivity pneumonitis: current concepts and future questions. J Allergy Clin Immunol 108(5):661–670

Reichenberger F, Kleiber B et al (2007) Bronchoalveolar lavage quality influences the T4/T8 ratio in sarcoidosis. Respir Med 101:2025–2030

Schuyler M, Cormier Y (1997) The diagnosis of hypersensitivity pneumonitis. Chest 111:534–536

Selman M, Lacasse Y et al (2010) Hypersensitivity pneumonitis caused by fungi. Proc Am Thorac Soc 7:229–236

Semenzato G (1991) Immunology of interstitial lung diseases: cellular events taking place in the lung of sarcoidosis, hypersensitivity pneumonitis and HIV infection. Eur Respir J 1:102

Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 23(5):581–590

Thomeer M, Demedts M (1997) Predictive value of CD4/CD8 ratio in bronchoalveolar lavage in the diagnosis of sarcoidosis [abstract]. Sarcoidosis Vasc Diffuse Lung Dis 14(Suppl 1):36

Winck JC, Delgado L et al (2004a) Antigen characterization of major cork moulds in suberosis (cork worker's pneumonitis) by immunoblotting. Allergy 59(7):739–745

Winck JC, Delgado L et al (2004b) Cork workers' occupational asthma: lack of association with allergic sensitisation to fungi of the work environment. Int Arch Occup Environ Health 77(4):296–300

Winterbauer R, Lammert J et al (1993) Bronchoalveolar lavage cell populations in the diagnosis of sarcoidosis. Chest 104:352–361

Ye Q, Nakamura S et al (2009) Interleukin 12, interleukin 18, and tumor necrosis factor alpha release by alveolar macrophages: acute and chronic hypersensitivity pneumonitis. Ann Allergy Asthma Immunol 102(2):149–154

Serum metalloproteinases 1 and 7 in the diagnosis of idiopathic pulmonary fibrosis and other interstitial pneumonias

António Morais, Marília Beltrão, Oksana Sokhatska, Diogo Costa, Natália Melo, Patrícia Mota, Agostinho Marques, Luís Delgado.

Respiratory Medicine (2015) 109, 1063-1068

Respiratory Medicine 109 (2015) 1063–1068

Contents lists available at ScienceDirect

Respiratory Medicine

journal homepage: www.elsevier.com/locate/rmed

Serum metalloproteinases 1 and 7 in the diagnosis of idiopathic pulmonary fibrosis and other interstitial pneumonias

António Morais [a, *], Marília Beltrão [b], Oksana Sokhatska [b], Diogo Costa [c, d], Natalia Melo [a], Patricia Mota [a], Agostinho Marques [a], Luís Delgado [b, e]

[a] Pneumology Department, Centro Hospitalar São João, Faculdade de Medicina, Universidade do Porto, Portugal
[b] Laboratory of Immunology, Basic and Clinical Immunology Unit, Faculdade de Medicina, Universidade do Porto, Portugal
[c] Department of Clinical Epidemiology, Predictive Medicine and Public Health, Faculdade de Medicina, Portugal
[d] EPIUnit-Institute of Public Health, Universidade do Porto, Portugal
[e] Centre for Research in Health Technologies and Information Systems (CINTESIS), University of Porto, Portugal

ARTICLE INFO

Article history:
Received 9 April 2015
Received in revised form
8 June 2015
Accepted 8 June 2015
Available online 12 June 2015

Keywords:
Metalloproteinases
Metalloproteinase 1
Metalloproteinase 7
Idiopathic pulmonary fibrosis
Idiopathic interstitial pneumonias
Sarcoidosis

ABSTRACT

Introduction: Accurate diagnosis of idiopathic pulmonary fibrosis (IPF) has important therapeutic and prognostic implications and would be greatly aided by reliable diagnostic biomarkers as IPF has sometimes overlapping features with other interstitial lung diseases (ILD).
Objectives: To explore the value of serum metalloproteinases (MMP) 1 and 7 levels in the differential diagnosis of IPF with other ILD.
Methods: MMP-1/7 serum levels were measured using Luminex xMAP technology in 139 patients- 47 IPF, 36 non-IPF Usual Interstitial Pneumonia (UIP), 14 idiopathic Nonspecific Interstitial Pneumonia (iNSIP), 29 secondary NSIP (secNSIP), 13 stage IV sarcoidosis- and 20 healthy controls, and compared using the Mann–Whitney U test.
Results: MMP-1 was significantly higher in IPF than non-IPF UIP ($P = .042$) and sarcoidosis ($P = .027$). MMP-7 was significantly higher in IPF than controls ($P < .001$), non-IPF UIP ($P = .003$), secNSIP ($P < .001$), and sarcoidosis ($P < .001$). The Area Under the Curve for IPF versus other ILD was 0.63 (95%CI, 0.53–0.73) for MMP-1, 0.73 (95%CI, 0.65–0.81) for MMP-7, and 0.74 (95%CI, 0.66–0.82) for MMP-1/MMP-7 combined. Sensitivity and specificity for MMP-7 cutoff = 3.91 ng/mL was 72.3% and 66.3%, respectively, Positive Predictive Values = 52.3% and Negative Predictive Values = 82.4%.
Conclusions: MMP-1 and particularly MMP-7 serum levels were significantly higher in IPF than in non-IPF UIP, the main entity in differential diagnosis. The value of these biomarkers as additional tools in a multidisciplinary approach to IPF diagnosis needs to be considered and further explored.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrosing lung disease characterized by progressive functional deterioration and a median survival of 2–3 years from the time of diagnosis [1–3]. It is associated with a radiological and histological pattern of usual interstitial pneumonia (UIP) and is the most common form of idiopathic interstitial pneumonia [1,4]. Although its pathogenesis is largely unknown, IPF is assumed to be caused by aberrant tissue repair and remodeling following recurrent alveolar epithelial injury (the nature of which is currently unknown), leading to a persistent and progressive disordered fibroproliferation [2,5,6]. Since the UIP pattern is not pathognomonic, IPF is always a diagnosis of exclusion, with ruling out of interstitial lung diseases related to connective tissue lung disorders (CTD-ILDs), hypersensitivity pneumonitis (HP), and drug lung toxicity [1,7,8]. Moreover, in cases without typical radiological UIP features (e.g., possible UIP pattern), it can be very difficult to distinguish between IPF and conditions such as idiopathic or secondary fibrotic nonspecific interstitial pneumonia (NSIP) [1,7]. An accurate diagnosis of IPF is of key importance considering the therapeutic and prognostic implications, and surgical lung biopsy is required in cases with an inconclusive diagnosis [1,7]. Biopsy, however, is an invasive procedure, with attendant risks (e.g, acute exacerbation in IPF), and

* Corresponding author. Pneumology Department, Centro Hospitalar São João, Faculdade de Medicina, Universidade do Porto, 4200-319, Portugal.
E-mail address: antonio.morais16@outlook.com (A. Morais).

http://dx.doi.org/10.1016/j.rmed.2015.06.003
0954-6111/

furthermore, it is not feasible in patients with severe disease or serious comorbidities [1,9,10].

Both clinicians and patients, therefore, would benefit greatly from reliable IPF diagnostic biomarkers. While several recent studies have identified and suggested a potential diagnostic or prognostic role for certain peripheral blood biomarkers (e.g., Krebs von den Lungen 6 antigen [KL-6], glycoprotein, surfactant proteins A and D, chemokine CCL-18, vascular endothelial growth factor, and glycoprotein YKL-40), none of these candidate markers has been validated or clinically accepted [11,12].

The matrix metalloproteinases (MMPs) belong to the zinc-dependent endoproteases that participate in extracellular matrix remodeling, wound healing, and angiogenesis and have been implicated in the pathogenesis of IPF [13,14]. Microarray studies have shown several members of the MMP family to be highly upregulated in IPF lungs, and elevated protein expression (MMP-1,2,3,7,8,9) has been found in the bronchoalveolar lavage fluid (BALF) and blood of IPF patients [13,15–20]. However, based on the available data, MMP-1 and MMP-7 are the most significantly overexpressed proteins in the lungs of patients with IPF compared with healthy controls [14]. Moreover, using a panel of 49 plasma proteins in a group of IPF patients, Rosas et al. [19] found increased concentrations of both MMP-1 and MMP-7, suggesting not only a determinant role in IPF pathogenesis but also potential utility as biomarkers in the differential diagnosis of this disease.

Although significant differences have been described for MMP-1 and MMP-7 expression in IPF compared with other ILDs, namely sarcoidosis and HP (mostly subacute forms), a direct comparison has never been made with conditions that pose the main challenge in the differential diagnosis of IPF [19]. The aim of our research was to compare serum MMP-1 and MMP-7 levels in IPF, non-IPF UIP, and fibrotic NSIP.

2. Material and methods

2.1. Patients and controls

We included 139 patients followed at the Interstitial Lung Diseases outpatient clinic at Centro Hospitalar São João, a tertiary referral center serving patients mostly from the north of Portugal. The patients were classified into 3 groups: IPF (n = 47), non-IPF UIP (n = 36), and fibrotic NSIP (n = 43). The non-IPF UIP group included 21 patients with HP (12 with bird fancier's disease, 6 with suberosis, and 3 with unknown etiology), 13 with CTD (7 with systemic sclerosis, 5 with rheumatoid arthritis, and 1 with undifferentiated CTD), and 2 with amiodarone lung toxicity. In the fibrotic NSIP group, there were 14 patients with idiopathic NSIP and 29 with NSIP secondary to an underling CTD (13 with systemic sclerosis, 8 with rheumatoid arthritis, 3 with Sjögren syndrome, 2 with mixed CTD, 2 with dermatopolymyositis, and 1 with systemic lupus erythematosus). Additionally, we included 13 patients with stage IV sarcoidosis and a control group of 20 healthy individuals (mean [SD] age of 70.3 [6.4] years, 9 females [45%]) with no history of lung disease or respiratory symptoms, or evidence of other diseases by chest radiography. Written informed consent was obtained from all individuals, and the study was approved by the hospital's ethics committee.

Patients with IPF were diagnosed according to the 2011 European Respiratory Society (ERS)/American Thoracic Society (ATS)/Japanese Respiratory Society/Latin American Thoracic Society Guidelines [1]. CTDs were diagnosed following The European League Against Rheumatism (EULAR) Recommendations [21]. All patients with NSIP underwent surgical lung biopsy, with application of the 2002 ATS/ERS International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias [22]. CTD-ILD diagnosis was based on high-resolution computed tomography (HRCT) findings, BALF features, and a previous CTD diagnosis [23]. HP was diagnosed using the criteria proposed by Lacasse et al. [24] Eleven (84.6%) of the 13 patients with sarcoidosis had histological confirmation in a compatible clinical and radiological context and the other 2 fulfilled criteria established in the consensus statement on sarcoidosis by the ERS/ATS/World Association of Sarcoidosis and Other Granulomatous Diseases (WASOG) [25], namely compatible clinical and radiographic features, a BALF CD4/CD8 lymphocyte ratio of >4.0, and a 2-year observation period to exclude other medical conditions. All radiological exams were evaluated by 2 radiologists and pathologic samples were analyzed by 2 pathologists, all trained in ILD evaluation. All the diagnoses were discussed and established by a multidisciplinary group including lung physicians, radiologists, and pathologists.

2.2. Blood samples

Blood samples were collected by venipuncture using Terumo Venosafe Serum-Gel tubes. Serum was separated within 30 min of blood collection following centrifugation for 10 min at 400× *g*. The serum samples were stored in aliquots at −80 °C until use.

MMP-1 and MMP-7 were measured using a human multiplex analysis assay (R&D Systems, Inc.) according to the manufacturer's protocol. Briefly, the assay is based on the Fluorokine MAP multiplex technology (Luminex Corporation), which combines the principle of a sandwich immunoassay with fluorescent-bead-based technology. Appropriate standards and serum samples were diluted (10-fold) in calibrator diluents and added to pre-wet filter-bottomed microplates. Fluorescent beads containing MMP-1 and MMP-7 antibodies were added and incubated on a plate shaker for 2 h at room temperature. After washing, biotinylated detection antibodies were added to each well, incubated for 1 h at room temperature, washed, and incubated for a further 30 min with a streptavidin-phycoerythrin conjugate. After washing, the beads were resuspended in wash buffer and analyzed on a Luminex 200 instrument. Mean fluorescent intensity data were analyzed using the Luminex 100 Integrated System version 2.3. The generation of a standard curve using standard MMP concentrations allowed the measurement of each metalloproteinase individually. The minimum detectable dose for MMP-1 and MMP-7 was 4.4 and 16.9 pg/mL, respectively.

2.3. Statistical analysis

Analysis of variance or Kruskal–Wallis tests (for nonnormally distributed variables) were used to compare continuous variables and the χ^2 test was used to compare proportions of demographic characteristics and MMP distributions according to diagnosis. The Mann–Whitney U test was used for pairwise comparison of the distribution of MMP-1 and MMP-7 serum levels in IPF patients versus controls and patients with other diagnoses. To calculate the associations of MMP-1 and MMP-7 independently with each disorder we computed logistic regression models adjusting for age, sex, and smoking history (never vs former or current smokers). A *P* value of less than 0.05 was considered significant and all *P* values were 2-sided.

Receiver operating characteristic (ROC) analysis was used to determine the area under the curve (AUC) for MMP-1, MMP-7, and MMP-1/MMP-7 combined with regard to IPF diagnosis versus any other diagnosis (excluding controls). Sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) were calculated for the cutoffs of MMP-1 and MMP-7, and the

Youden index (J = max[sensitivity + specificity−1]) was used to establish the best cutoff for IPF diagnosis.

3. Results

Patient demographics and characteristics according to diagnosis are presented in Table 1, together with statistically significant between-group differences based on the expected clinical characteristics of each group [21]. Healthy controls and IPF patients had a similar mean (SD) age (70.3 [6.4] vs 70.6 [9.5] years, $P = .865$) and sex distribution (9 females [45.0%] vs 17 [36.2%], $P = .686$) and similar proportions of smokers (11 [55.0%] vs 28 [62.2%], $P = .583$).

A significant difference was found for the mean serum concentration of MMP-7 but not MMP-1 across the groups (Table 1). MMP-1 concentration, however, was significantly higher in IPF than in non-IPF UIP ($P = .042$) and sarcoidosis ($P = .027$) (Fig. 1). MMP-7 levels were significantly higher in IPF patients than in controls ($P < .001$) and patients with non-IPF UIP ($P = .003$), secondary NSIP ($P < .001$), and sarcoidosis ($P < .001$) (Fig. 2).

In the multivariate logistic regression models for each diagnosis (Table 2), MMP-7 was independently associated with IPF (OR = 1.34; 95% CI, 1.17–1.54) and an inverse association was found for sarcoidosis (OR = 0.66; 95% CI, 0.45–0.96), ie, lower levels of MMP-7 were associated with the diagnosis of sarcoidosis. The association between MMP-7 and IPF remained significant after adjusting for sex, age and smoking history. No further significant associations were observed.

The AUC of the ROC curve for the diagnosis of IPF versus all other diagnoses was 0.63 (95% CI, 0.53–0.73) for MMP-1, 0.73 (95% CI, 0.65–0.81) for MMP-7, and 0.74 (0.66–0.82) for MMP-1 and MMP-7 combined (Fig. 3)

The best combination of sensitivity and specificity for MMP-7 was obtained for the cutoff of 3.91 ng/mL (72.3% and 66.3%, respectively), with a PPV of 52.3% and a NPV of 82.4% (Supplementary Table). As lower cutoffs were selected, higher NPVs were achieved, demonstrating better performance for each MMP in ruling out IPF.

4. Discussion

We evaluated the potential value of MMP-1 and MMP-7 as diagnostic biomarkers for IPF by comparing serum concentrations in IPF patients and patients with other ILDs that pose a challenge in the differential diagnosis (non-IPF UIP and secondary and idiopathic fibrotic NSIP). Both MMP-1 and MMP-7 serum levels were significantly higher in IPF patients than in patients with non-IPF UIP and sarcoidosis. Moreover, MMP-7 levels were significantly higher in IPF patients than in patients with secondary NSIP and healthy controls (with a similar age, sex distribution and smoking habits). MMP-7 performed better than MMP-1 for the diagnosis of IPF, as measured by the AUC, with a cutoff of 3.91 ng/mL providing maximum sensitivity and specificity and an NPV of 82.4%.

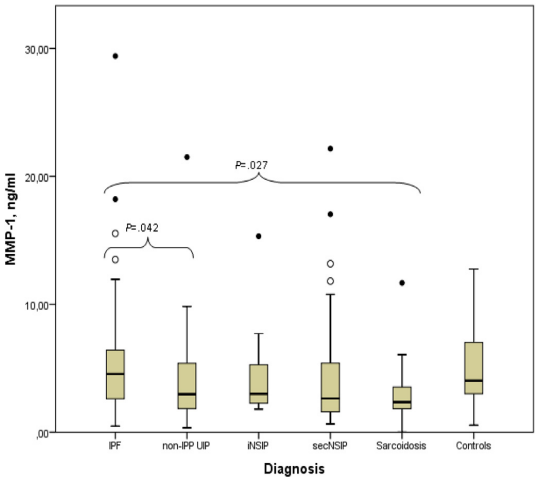


Fig. 1. White circles represent mild outliers and black circles represent extreme outliers. MMP-1 levels in patients and controls. Levels were significantly higher in IPF than in non-IPF UIP ($P = .042$) and sarcoidosis ($P = .027$). MMP indicates matrix metalloproteinase; IPF, idiopathic pulmonary fibrosis; non-IPF UIP, non-IPF usual interstitial pneumonia; iNSIP, idiopathic nonspecific interstitial pneumonia; secNSIP, NSIP secondary to a connective tissue lung disorder; UIP, usual interstitial pneumonia.

IPF is the most severe of the idiopathic interstitial pneumonias, with a distinctive therapeutic approach and a poor prognosis. Consequently, a confident diagnosis and a clear distinction from other fibrotic ILDs have important clinical implications in terms of patient outcome and treatment options. Approximately one-third of IPF patients require surgical lung biopsy, which has inherent risks and is not always feasible [1]. Clinicians, therefore, need biomarkers not only to predict outcome and survival, but also to establish a reliable diagnosis.

An ideal biomarker should be easily accessible, amenable to reliable measurement, and suitable for use in longitudinal assessment [11,12]. Increasing evidence is being amassed on the potential value of MMPs in IPF [14,16]. Rosas et al. [19] analyzed 49 potential IPF serum markers and found 5 MMPs (including MMP-1 and MMP-7) among the 12 proteins that were differentially expressed. Moreover, MMP-1 and MMP-7 were significantly overexpressed in IPF patients compared with patients with sarcoidosis or chronic obstructive pulmonary disease (COPD), and enhanced MMP-7 expression was seen in both lung tissue and BALF.

In addition to experimental data [26,27], several investigations with lung tissue, BALF, and peripheral blood support a possible role of MMPs in the pathogenesis of IPF, namely through their

Table 1
Patients characteristics according to diagnosis.

	UIP		NSIP		Sarcoidosis (n = 13)	Controls (n = 20)	*P* Value[a]
	IPF (n = 47)	Non-IPF (n = 36)	iNSIP (n = 14)	secNSIP (n = 29)			
Age, mean (SD), y	70.6 (9.5)	65.4 (11.5)	62.4 (12.1)	61.1 (9.5)	53.9 (12.6)	70.3 (6.4)	<.001
Female, No. %	17 (36.2)	24 (66.7)	10 (71.4)	26 (89.7)	7 (53.8)	9 (45.0)	<.001
Ex/current smokers, No. (%)	28 (62.2)	5 (13.9)	4 (28.6)	8 (27.6)	4 (30.8)	11 (55.0)	<.001
Never smokers, No. (%)	17 (37.8)	31 (86.1)	10 (71.4)	21 (72.4)	9 (69.2)	9 (45.0)	
MMP-1, mean (SD), ng/mL	5.79 (5.20)	4.02 (3.85)	4.43 (3.59)	4.93 (5.25)	3.22 (2.96)	4.89 (3.11)	.200
MMP-7, mean (SD), ng/mL	5.79 (3.07)	4.07 (2.73)	4.32 (2.90)	3.76 (2.62)	2.39 (1.68)	1.52 (0.77)	<.001

Abbreviations: iNSIP, idiopathic NSIP; MMP, matrix metalloproteinase; NSIP, nonspecific interstitial pneumonia; secNSIP, NSIP secondary to a connective tissue lung disorder; UIP, usual interstitial pneumonia.
[a] Comparing all groups.

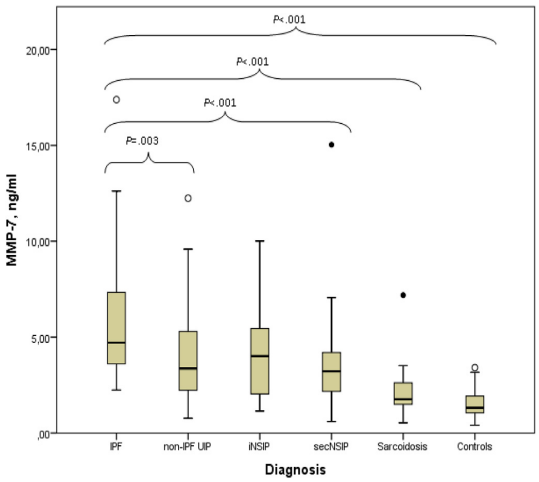


Fig. 2. White circles represent mild outliers and black circles represent extreme outliers. MMP-7 levels in patients and controls. Levels were significantly higher in IPF patients than in controls ($P < .001$), patients with non-IPF UIP ($P = .003$), secondary NSIP ($P < .001$), and sarcoidosis ($P < .001$). MMP indicates matrix metalloproteinase; IPF, idiopathic pulmonary fibrosis; non-IPF UIP, non-IPF usual interstitial pneumonia; iNSIP, idiopathic nonspecific interstitial pneumonia; secNSIP, NSIP secondary to a connective tissue lung disorder; UIP, usual interstitial pneumonia.

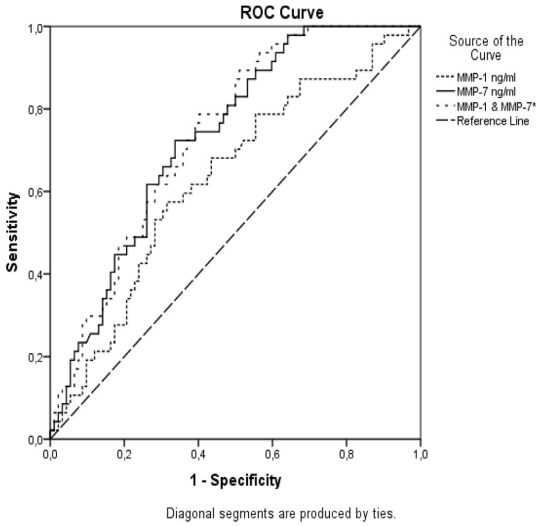


Fig. 3. Receiver operator characteristic (ROC) curves for MMP-1 and MMP-7 comparing idiopathic pulmonary fibrosis and all other diagnoses. *The combined variable (MMP-1 & MMP-7) is the predicted probability extracted from a logistic regression model fitted for IPF diagnosis vs other, using MMP-1 and MMP-7 as covariates.

contribution to extracellular matrix remodeling. In fact, MMPs are involved not only in the degradation of matrix components, but also in the modulation of a variety of bioactive mediators, such as cytokines and chemokines [13,14]. MMPs are regulated at transcriptional and posttranscriptional levels by inhibitors and inducers, creating a delicate balance in extracellular matrix remodeling [13,14]. Their expression, usually modest under physiological conditions, increases in repair processes such as wound healing, and uncontrolled MMP activity results in tissue damage and functional changes [14].

MMP-7, the smallest member of the MMP family, has a profibrotic profile with diverse biological functions, ranging from innate immunity to inflammation, apoptosis, and fibroproliferation [27,28]. It is also able to process numerous bioactive substrates and activate proteases, including itself. The release of preformed TGF-β from extracellular matrix is the main regulation process related to TGF bioactivity, one of the presumptive mediators in IPF pathogenesis. In an animal model, MMP-7 knockout mice treated with bleomycin did not develop lung fibrosis [29]. Additionally, MMP-7 has been found on the surface of epithelial cells and alveolar macrophages in IPF lung tissue, but not in healthy lung tissue [30,31]. Interestingly, enhanced levels of serum MMP-7 were also found in patients with asymptomatic IPF (although at lower levels than in symptomatic patients), pointing to its possible value as a marker for both early disease and progression [28]. In fact, BALF and serum MMP-7 levels showed not only a negative correlation with forced vital capacity and diffusing capacity but also an independent association with IPF mortality [3,19,20,31].

The potential role of MMP-7 as a differential biomarker in IPF is still unclear, particularly when clinically similar ILDs are involved. In fact, Rosas et al. compared IPF with sarcoidosis and COPD, which clearly have distinct clinical and radiological features. Although the study included patients with HP mostly with subacute presentation, the major confounding diagnosis is chronic advanced HP associated with fibrosis, particularly with UIP pattern. Our study, which showed significantly higher serum MMP-7 levels in IPF compared with non-IPF UIP, is the first, to our knowledge, to investigate the value of this marker in distinguishing IPF from other ILDs with the UIP pattern.

Vuorinen et al. [18] described a higher level of MMP-7 in the BALF of IPF patients compared with controls, with no significant differences for idiopathic NSIP or sarcoidosis. In our series, while we did not find significant differences for serum MMP- 7 levels between IPF and idiopathic NSIP, we did observe significantly

Table 2
Odds ratios (95% CI) for the association of MMP-1 and MMP-7 with each diagnosis.

	IPF	Non-IPF UIP	NSIP	secNSIP	Sarcoidosis
MMP-1a	1.07 (0.99–1.15)	0.94 (0.85–1.04)	0.98 (0.85–1.12)	1.01 (0.92–1.10)	0.86 (0.68–1.08)
MMP-1 adjusted[b]	1.04 (0.96–1.13)	0.95 (0.85–1.05)	1.00 (0.88–1.14)	1.06 (0.97–1.15)	0.88 (0.68–1.13)
MMP-1 adjusted[c]	1.05 (0.97–1.14)	0.94 (0.85–1.04)	1.00 (0.88–1.14)	1.06 (0.96–1.15)	0.88 (0.69–1.12)
MMP-7	1.34 (1.17–1.54)[a]	1.00 (0.88–1.13)	1.03 (0.86–1.23)	0.95 (0.82–1.10)	0.66 (0.45–0.96)[a]
MMP-7 adjusted[b]	1.39 (1.18–1.63)[a]	1.00 (0.87–1.14)	1.06 (0.88–1.28)	1.00 (0.85–1.17)	0.72 (0.48–1.08)
MMP-7 adjusted[c]	1.40 (1.18–1.66)[a]	1.00 (0.87–1.14)	1.06 (0.88–1.28)	1.00 (0.85–1.17)	0.73 (0.49–1.09)

Abbreviations: IPF, idiopathic pulmonary fibrosis; MMP, matrix metalloproteinase; NSIP, nonspecific interstitial pneumonia; secNSIP, NSIP secondary to a connective tissue lung disorder; UIP, usual interstitial pneumonia.

[a] $P < .05$.

[b] Adjusted for age and sex.

[c] Adjusted for age, sex, and smoking status (smoking status variable was dichotomized: former or current smokers vs never smokers).

higher levels in IPF than in NSIP with underlying CTD. Interestingly, similar gene expression patterns have been found in IPF and some NSIP cases, but there are clear differences when other ILDs that can nevertheless express an UIP or NSIP pattern are considered [15]. Moreover, all the patients with idiopathic NSIP included had thoracic HRCT features alike UIP instead of HP or organizing pneumonia like features, the two other possible patterns described in this entity. Vuorinen et al. showed lung MMP-7 immunoreactivity in some fibroblastic foci, vascular smooth muscle cells, and areas of hyperplastic epithelium in IPF samples; in NSIP samples immunoreactivity was observed in areas of inflammation beneath the alveolar epithelium. These findings suggest that MMP-7 may have relevant but different roles in the pathophysiologic processes involved in different ILDs, but the differences might not be quantitatively detectable. Moreover, although idiopathic NSIP is associated with a better prognosis, it is sometimes difficult to clearly distinguish from IPF on HRCT. Furthermore, the 2 entities have a similar BALF profile and some patients with IPF show NSIP areas in their surgical biopsy. The above observations suggest that IPF and NSIP may possibly be connected.

Our results concerning sarcoidosis contrast with those of Vuorinen et al. [18] since we found significant low MMP-7 levels in comparison with IPF. They are in agreement with Rosas et al. [19] and Zhou et al. [32] findings, but our study is the first to include only sarcoidosis patients with lung fibrosis. The majority of patients studied by Vuorinen et al. did not have significant lung disease, while those studied by Zhou et al. had stage II sarcoidosis. In another study, Huh et al. [33] found no significant differences in BALF MMP-7 levels between patients with IPF and cryptogenic organizing pneumonia (COP) although it included few patients and COP is rarely confused with UIP.

Like MMP-7, MMP-1 has multiple biological functions that may be involved in the pathogenesis of IPF, such as cytokine processing, cell migration and growth regulation. MMP-1 expression in IPF seems to be primarily in the alveolar epithelium and is not observed in fibroblastic foci or in the interstitial compartment, where interstitial collagens are secreted and accumulated, respectively [13,28]. It has also been suggested that MMP-1 might contribute to the formation of cystic spaces, resulting in the characteristic honeycombing pattern seen in IPF [14]. MMP-1 has been shown to be upregulated in the lung tissue of IPF compared to HP patients and healthy controls [15,34]. To our knowledge, ours is the first report to show significantly higher serum MMP-1 levels in IPF compared with non-IPF UIP. The significantly lower MMP-1 levels detected in sarcoidosis are consistent with the findings of Rosas et al. [19].

The relatively small sample size for some of the clinical entities considered in our analysis, particularly idiopathic NSIP, is the main limitation of this study. The cutoff values used were based on the distribution of MMPs in this particular sample of patients and thus could vary in other groups. Replications of our findings are therefore needed. While it is largely believed that a combination of biomarkers has a greater likelihood of becoming a reliable diagnostic tool for IPF than any single biomarker, combinations can be difficult to implement for methodological and economic reasons. Our data show that MMP-7 is clearly the best differentiator but, unlike previous studies, we found that its specificity improved only marginally when MMP-1 was added.

In conclusion, our results provide additional support for the potential value of serum MMP-1 and MMP-7 concentrations as diagnostic biomarkers in IPF. Since non-IPF UIP poses the main challenge in the differential diagnosis of IPF, the implications of significantly higher levels of MMP-1 and, in particular, MMP-7 in IPF patients need to be carefully considered as potential tools in the multidisciplinary diagnostic approach to IPF.

Authors contribution

AM, LD and AM elaborated the study conception and design, analysis of data, manuscript preparation and revision.

MB and OS performed the laboratory proceedings.

NM and PM assisted the patients in the Interstitial Lung Diseases unit, cooperated in the data acquisition and drafted the manuscript.

DC participated in the data acquisition and performed the statistic evaluation.

All authors read and approved the final manuscript.

Conflict of interests statement

The authors declare that they do not have any conflict of interests to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.rmed.2015.06.003.

References

[1] An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence based guidelines for diagnosis and management, Am. J. Respir. Crit. Care Med. 183 (2011) 788–824.
[2] T.E. King Jr., A. Pardo, M. Selman, Idiopathic pulmonary fibrosis, Lancet 378 (2011) 1949–1961.
[3] R. Putman, I. Rosas, G. Hunninghake, Genetics and early detection in idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care Med. 189 (2014) 770–778.
[4] B.T. Larsen, T.V. Colby, Update for pathologists on idiopathic interstitial pneumonias, Arch. Pathol. Lab. Med. 136 (2012) 1234–1241.
[5] M. Selman, T.E. King, A. Pardo, Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy, Ann. Intern. Med. 134 (2) (2001) 136–151.
[6] D.F. Zoz, W.E. Lawson, T.S. Blackwell, Idiopathic pulmonary fibrosis: a disorder of epithelial cell dysfunction, Am. J. Med. Sci. 341 (2011) 435–438.
[7] A.U. Wells, N. Hirani, Interstitial lung disease guideline, Thorax 63 (2008) v1–v58.
[8] A. Caminati, S. Harari, IPF: new insight in diagnosis and prognosis, Respir. Med. 104 (2010) S2–S10.
[9] Riitta Kaarteenaho, The current position of surgical lung biopsy in the diagnosis of idiopathic pulmonary fibrosis, Respir. Res. 14 (2013) 43.
[10] M. Blanco, G.A. Obeso, J.C. Durán, J.E. Rivo, E. García-Fontán, E. Peña, M. Rodríguez, J. Albort, M.A. Cañizares, Surgical lung biopsy for diffuse lung disease. Our experience in the last 15 years, Rev. Port. Pneumol. 19 (2013) 59–64.
[11] Y. Zhang, N. Kaminski, Biomarkers in idiopathic pulmonary fibrosis, Curr. Opin. Pulm. Med. 18 (5) (2012 Sep) 441–446.
[12] R. Vij, I. Noth, Peripheral blood biomarkers in idiopathic pulmonary fibrosis, Transl. Res. 159 (4) (2012 Apr) 218–227.
[13] R.C.A. Dancer, A.M. Wood, D.R. Thickett, Metalloproteinases in idiopathic pulmonary fibrosis, Eur. Respir. J. 38 (2011) 1461–1467.
[14] A. Pardo, M. Selman, Role of matrix metaloproteases in idiopathic pulmonary fibrosis, Fibrogenesis Tissue Repair 5 (Suppl. 1) (2012) S9.
[15] M. Selman, A. Pardo, L. Barrera, A. Estrada, S.R. Watson, K. Wilson, N. Aziz, N. Kaminski, A. Zlotnik, Gene expression profiles distinguish idiopathic pulmonary fibrosis from hypersensitivity pneumonitis, Am. J. Respir. Crit. Care Med. 173 (2006) 188–198.
[16] S. McKeown, A.G. Richter, C.O. Kane, D.F. McAuley, D.R. Thickett, MMP expression and abnormal lung permeability are important determinants of outcome in IPF, Eur. Respir. J. 33 (2009) 77–84.
[17] M. Suga, K. Iyonaga, T. Okamoto, Y. Gushima, H. Miyakawa, T. Akaike, M. Ando, Characteristic elevation of matrix metalloproteinase activity in idiopathic interstitial pneumonias, Am. J. Respir. Crit. Care Med. 162 (2000) 1949–1956.
[18] K. Vuorinen, M. Myllarniemi, L. Lammi, P. Piirilä, P. Rytilä, K. Salmenkivi, V.L. Kinnula, Elevated matrilysinlevels in bronchoalveolar lavage fluid do not distinguish idiopathic pulmonary fibrosis from other interstitial lung diseases, APMIS 115 (2007) 969–975.
[19] I.O. Rosas, T.J. Richards, K. Konishi, Y. Zhang, K. Gibson, A.E. Lokshin, K.O. Lindell, J. Cisneros, S.D. Macdonald, A. Pardo, F. Sciurba, J. Dauber, M. Selman, B.R. Gochuico, N. Kaminski, MMP1 and MMP7 as potential peripheral blood biomarkers in idiopathic pulmonary fibrosis, PLoS Med. 5 (2008) e93.
[20] T.J. Richards, N. Kaminski, F. Baribaud, S. Flavin, C. Brodmerkel, D. Horowitz, K. Li, J. Choi, L.J. Vuga, K.O. Lindell, M. Klesen, Y. Zhang, K.F. Gibson, Peripheral blood proteins predict mortality in idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care Med. 185 (1) (2012) 67–76.

[21] http://www.eular.org/index.cfm?framePage=/recommendations_home.cfm.
[22] American Thoracic Society; European Respiratory Society, American thoracic society/European respiratory society international multidisciplinary consensus classification of the idiopathic interstitial pneumonias, Am. J. Respir. Crit. Care Med. 165 (2) (2002) 277–304.
[23] A. Fisher, R. du Bois, Interstitial lung disease in connective tissue disorders, Lancet 380 (9842) (2012) 689–698.
[24] Y. Lacasse, M. Selman, U. Costabel, J.C. Dalphin, M. Ando, F. Morell, R. Erkinjuntti-Pekkanen, N. Muller, T.V. Colby, M. Schuyler, Y. Cormier, Clinical diagnosis of hypersensitivity pneumonitis, Am. J. Respir. Crit. Care Med. 168 (8) (2003 Oct 15) 952–958.
[25] Statement on sarcoidosis. Joint statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999, Am. J. Respir. Crit. Care Med. 160 (2) (1999) 736–755.
[26] X.S. Puente, L.M. Sanchez, C.M. Overall, C. López-Otín, Human and mouse proteases: a comparative genomic approach, Nat. Rev. Genet. 4 (2003) 544–558.
[27] G.A. Limb, K. Matter, G. Murphy, A.D. Cambrey, P.N. Bishop, G.E. Morris, P.T. Khaw, Matrix metalloproteinase-1 associates with intracellular organelles and confers resistance to lamin A/C degradation during apoptosis, Am. J. Pathol. 166 (2005) 1555–1563.
[28] A. Pardo, M. Selman, N. Kaminski, Approaching the degradome in idiopathic pulmonary fibrosis, Int. J. Biochem. Cell. Biol. 40 (2008) 1141–1155.
[29] F. Zuo, N. Kaminski, E. Eugui, J. Allard, Z. Yakhini, A. Ben-Dor, L. Lollini, D. Morris, Y. Kim, B. DeLustro, D. Sheppard, A. Pardo, M. Selman, R.A. Heller, Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans, Proc. Nat. Acad. Sci. U. S. A. 99 (2002) 6292–6297.
[30] S. Fujishima, T. Shiomi, S. Yamashita, Y. Yogo, Y. Nakano, T. Inoue, M. Nakamura, S. Tasaka, N. Hasegawa, N. Aikawa, A. Ishizaka, Y. Okada, Production and activation of matrix metalloproteinase 7 (matrilysin 1) in the lungs of patients with idiopathic pulmonary fibrosis, Arch. Path Lab. Med. 134 (2010) 1136–1142.
[31] J.W. Song, K.H. Do, S.J. Jang, T.V. Colby, S. Han, D.S. Kim, Blood biomarkers MMP-7 and SP, Chest 143 (5) (2013) 1422–1429.
[32] L.F. Zhou, L. Jiang, Z.H. Li, J. Kang, Change of matrix metalloproteinase-1 and matrix metalloproteinase-7 in serum and bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and sarcoidosis, Zhonghua Jie He He Hu Xi Za Zhi 33 (2010) 441–444.
[33] J.W. Huh, D.S. Kim, Y.M. Oh, T.S. Shim, C.M. Lim, S.D. Lee, Y. Koh, W.S. Kim, W.D. Kim, K.R. Kim, Is metalloproteinase-7 specific for idiopathic pulmonary fibrosis? Chest 133 (2008) 1101–1106.
[34] K. Konishi, K.F. Gibson, K.O. Lindell, T.J. Richards, Y. Zhang, R. Dhir, M. Bisceglia, S. Gilbert, S.A. Yousem, J.W. Song, D.S. Kim, N. Kaminski, Gene expression profiles of acute exacerbations of idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care Med. 180 (2009) 167–175.

Discussão

FATORES IMUNOGENÉTICOS NA SUSCETIBILIDADE E EVOLUÇÃO DA SARCOIDOISE (ESTUDOS I, II E III)

A visão atual da patogénese da sarcoidose aponta para uma interação de um conjunto diversificado de genes de suscetibilidade em prováveis fatores ambientais ainda não identificados.[46, 49, 52, 147, 150] É também de admitir que essa interação genética seja igualmente decisiva na heterogeneidade quer da apresentação quer do tipo de evolução clínica desta doença.[54, 146, 148, 151, 155, 161] Torna-se pois necessária a investigação destas relações/cooperações genéticas, muitas delas influenciando pontos críticos da resposta imunológica, dado poderem ficar evidentes determinados perfis de suscetibilidade e de evolução clínica, podendo nesse caso proporcionar no futuro estratégias terapêuticas mais adequadas ao perfil de cada doente.

De fato, os genes que mais frequentemente têm sido implicados no contexto da sarcoidose, são os relacionados com a apresentação e reconhecimento antigénico aos linfócitos T (como é o HLA), assim como algumas citocinas e seus receptores.[146-148, 151, 160-162] Os alelos do HLA, nomeadamente da classe II, têm sido os mais investigados, encontrando-se na literatura várias descrições em diferentes populações, o que é importante dada a variabilidade étnica associada. Os alelos HLA-*DRB1*0301* e HLA-*DQB1*0201* e o haplótipo HLA-*DRB1*0401-DPB1*0401* foram associados a um prognóstico favorável e resolução da doença.[206-214] Contrariamente, os alelos HLA-*DRB1*12*, HLA-*DRB1*1401*, HLA-*DRB3*0101* e HLA-*DQB1*0602*, para além do haplótipo HLA-*DRB1*1501-DQB1*0602* tem sido associados à evolução para formas crónicas.[209-213, 215] Num grupo de doentes com sarcoidose originários do norte de Portugal, concluímos que apenas o alelo HLA-*DRB1*03* se encontra associado à evolução da doença (após a correcção *Bonferroni* para múltiplas comparações), nomeadamente com a resolução da doença. Esta associação mantém-se mesmo após serem retirados da amostra os doentes com síndrome de *Löfgren*, dada a associação deste fenótipo a este alelo e a um bom prognóstico, motivo pelo qual poderia exercer um efeito confundidor.

Outras regiões genéticas relacionadas com a resposta imunitária têm igualmente sido objecto de investigação, ganhando particular destaque os polimorfismos do *BTNL2*, nomeadamente o alelo *rs2076530, o* mais

frequentemente associado à sarcoidose noutras séries(166-170, 216-219) O *BTNL2* é um membro das *butyrophilin-like molecules* (BTNLs) com homologia estrutural à família de moléculas de co-estimulação dos linfócitos T CD80/CD86 (B7), que são ambas expressas em células apresentadoras de antigénio (APCs), como são as células dendríticas nos órgãos linfóides periféricos (gânglios e baço).[163, 164] Foi também demonstrado que o *BTNL2* inibe a proliferação de células T e a sua produção de IL-2 e de citocinas pró-inflamatórias.[163-165] O polimorfismo que estudamos resulta num gene *BTNL2* mutado, tendo como consequências a produção de uma proteína truncada e a perturbação da sua inserção na membrana celular.[220] Não estando ainda estudadas as consequências funcionais desta alteração, é possível que a expressão alterada em APCs de uma molécula que induz sinalização negativa aos linfócitos T possa resultar numa elevada ativação e proliferação destes, uma das alterações imunopatológicas que se verificam na sarcoidose.[163, 221, 222] A descrição feita por *Wijnen et al.* de uma associação adicional do *BTNL2 rs2076530* A com a doença crónica persistente, poderá suportar um papel funcional deste polimorfismo na perda de um mecanismo de regulação do processo imunopatológico da sarcoidose.[170]

Desde a primeira descrição da associação do SNP *BTNL2 rs2076530* A, e independente da influência de alelos HLA, com a suscetibilidade à sarcoidose numa população germânica, tem surgido estudos que sustentam esta associação.[166-170, 216, 218, 219] *Spagnolo et al.* encontraram esta associação numa coorte de doentes originários de Inglaterra e Holanda, que no entanto desaparecia após exclusão dos doentes com síndroma de *Löfgren* e ajustamento para os alelos HLA-*DRB1*.[167] *Rybicki et al.* voltaram a encontrar a associação do SNP *rs2076530* numa população caucasiana da América do Norte, não tendo curiosamente sido verificada a mesma associação numa população negra da mesma origem.[166] Este estudo voltou a comprovar uma influência decisiva da raça no risco imunogenético, uma vez que a associação do *BTNL2* era independente dos alelos da classe II do HLA, mas com influência oposta nos Afro-americanos. Por outro lado *Milman et al.* descreveram a mesma associação numa população originária da Dinamarca, embora não tenha sido feito o estudo de desequilíbrio de ligação com os alelos HLA de classe II.[216]

Numa muito recente revisão sistemática da associação do gene *BTNL2* com a sarcoidose e que inclui o nosso estudo I, concluiu, a partir de 3303 casos e 2514 controlos já estudados em 7 regiões europeias, Japão e EUA, que o SNP *BTNL2 rs2076530* contribui para o risco de sarcoidose.[223]

Para além da suscetibilidade e das diferentes expressões fenotípicas, existem descrições acerca da eventual influência dos polimorfismos do gene *BTNL2* com a evolução na sarcoidose.[125, 169, 170, 214, 220] *Li et al.* reportaram a associação do SNP *rs2076530* G/A com o curso crónico da doença numa população germânica, enquanto *Coudurier et al.* relataram a associação deste mesmo SNP em três doentes com formas graves da doença.[169, 220] Contudo, em nenhum destes estudos foi feito a avaliação de desequilíbrio

de ligação com os alelos HLA da classe II. No entanto, em linha com estes resultados, *Wijnen et al.* relataram numa população de doentes holandeses uma associação do *BTNL2 rs2076530* A com a doença crónica persistente em comparação com a doença crónica estável, independentemente da influência dos genes *DRB1*.[170] Efetivamente, nos doentes com sarcoidose que evoluem para a cronicidade, observam-se casos em que se verificam apenas alterações imagiológicas e sem tradução funcional significativa até situações de insuficiência respiratória grave.[224] A diferenciação através de polimorfismos genéticos subjacentes entre doentes com formas crónicas e estáveis e outros que eventualmente exijam uma maior intervenção terapêutica torna-se, de fato, pertinente.[170, 224] Mais recentemente, *Wennerstrom et al.* reportaram igualmente a associação do SNP *rs2076530* A com a doença crónica persistente, tendo no entanto usado uma comparação com o grupo controlo e não com o grupo de doentes crónicos estáveis (como no estudo prévio de *Wijnen et al.*)[214]. No nosso coorte de doentes originários do Norte de Portugal (estudo I), não foi possível estabelecer uma associação do SNP *BTNL2 rs2076530* A com a evolução para a cronicidade, uma vez que não encontramos nenhuma associação estatisticamente significativa nem mesmo quando dividimos esse subgrupo em doentes com doença crónica estável e com doença crónica persistente.

No cromossoma 10q22.3 foi entretanto identificado outro gene, *ANXA 11*, com um polimorfismo fortemente associado à sarcoidose (SNP *rs1049550* C/T).[171] A anexina A11 é expressa numa grande diversidade de tecidos e células imunitárias, com uma expressão elevada detetada no pulmão e músculo liso, sugerindo o seu envolvimento em mecanismos celulares essenciais.[171, 225] A mutação estudada está localizada no exão 6 e resulta numa substituição de uma Citosina por uma Timidina (C/T), que altera o codão de uma arginina básica para uma cisteína polar no resíduo 230 (R230C) sendo que esta alteração, muito provavelmente, a estrutura da proteína que codifica ou interfere com a sua função.[175, 225, 226] Apesar do seu significado funcional na sarcoidose não estar estabelecido, diferentes estudos experimentais têm revelado que a anexina A11 tem um papel na regulação da proliferação e apoptose celular, pois a sua inativação diminui a proliferação e aumenta a apoptose celular.[172, 175, 176] É possível que uma disfunção da anexina A11 possa afetar vias da apoptose e induzir um desequilíbrio entre a apoptose e a sobrevida das células inflamatórias ativadas.[227]

Após a primeira descrição da associação do SNP *rs1049550* C/T como a associação mais significativa com a sarcoidose por *Hoffman et al.*, esta associação protetora foi confirmada por *Li et al.* igualmente numa população germânica.[174] Posteriormente, a diminuição significativa do *ANXA 11 rs1049550* C/T em doentes com sarcoidose comparativamente com grupo controlo foi observado numa população checa e numa população norte-americana, curiosamente quer no grupo que englobava indivíduos caucasianos, quer no grupo relativo a afro-americanos.[172, 173] O nosso

trabalho (estudo II) confirmou uma vez mais esta associação, com a particularidade do efeito protetor do alelo *rs1049550**T aumentar com o seu número de cópias no genótipo (um efeito gene-dose). No entanto, no nosso estudo o efeito protetor do alelo T verifica-se apenas em doentes sem a apresentação com o síndrome de *Löfgren*, em contraste com os achados de *Mrazek et al* na população checa. Já posteriormente à nossa publicação, a associação protectora do *ANXA 11 rs1049550* C/T foi igualmente verificada numa população chinesa, o que sugere esta associação ser etnicamente independente, dado que para além de caucasianos, existe a descrição de resultados semelhantes num grupo de afro-americanos e noutro de chineses.[173, 228]

Relativamente à evolução clínica, não foi publicado até ao momento qualquer associação com este polimorfismo. No entanto *Mzarek et al* e *Feng et al.* descreveram a associação com o estadio I de envolvimento torácico, casos habitualmente associados a resolução da doença, muitas vezes de forma espontânea.[172, 228] Contrariamente, *Li et. al* referiram não ter encontrado associações com nenhum dos tipos de evolução e *Levin et al* descreveu uma associação com o estadio IV de *Scadding*, equivalente a doença crónica, mas apenas em Afro-americanos.[173, 174] No nosso estudo, apesar de termos encontrado um aumento da frequência do genótipo TT da anexina A11 em doentes com neutrofilia no LBA, achado habitualmente descrito como sendo um marcador de cronicidade, não obtivemos nenhuma associação com a regressão ou com a evolução crónica (mesmo considerando formas crónicas estáveis e persistentes) e tendo igualmente em conta 2 e 5 anos de evolução.

A sarcoidose, como provavelmente muitas das doenças do interstício pulmonar, é uma doença multifatorial, com potenciais interações gene-gene e gene-ambiente.

Baseados nos resultados sobre as associações genéticas publicados e que estudamos, quisemos investigar uma possível interacção gene-gene entre os alelos das classes I e II do HLA, o *BTNL2 rs2076530* G/A e o *ANXA 11 rs2076530* G/A (Estudo III). Verificamos que apenas o *DRB1*03* se encontrava associado com a evolução da doença, mais concretamente com a regressão da doença, não se encontrando nenhuma interação significativa entre os alelos HLA e os SNPs estudados, nos modelos de regressão logística aplicados. Assim, genes ligados à suscetibilidade não parecem associar-se, necessariamente, à evolução clínica das doenças. Apesar de termos investigado as influências imunogenéticas com maior evidência na atualidade, é possível que outros genes mais ligados à evolução da doença ainda não tenham sido encontrados e descritos.

SUBPOPULAÇÕES LINFOCITÁRIAS CD103⁺ NA SARCOIDOISE E PNEUMONITE DE HIPERSENSIBILIDADE (ESTUDOS IV E V)

A sarcoidose é caraterizada por uma linfocitose alveolar de predominio $CD4^+$, as células efetoras envolvidas numa resposta imunitária Th1 e, consequentemente, por uma razão $CD4^+/CD8^+$ elevada no BAL.[45, 46, 63, 150] No entanto, uma linfocitose $CD4^+$ pode também ser observada noutras doenças do interstício pulmonar, sendo que a sensibilidade diagnóstica de uma razão $CD4^+/CD8^+ > 3,5$ na sarcoidose raramente ultrapassa os 50% na maior parte dos estudos publicados.[62, 63, 229] Neste contexto a integrina CD103, expressa em linfócitos intraepiteliais nas superfícies mucosas tem sido estudada, verificando-se que a proporção relativa de linfócitos T no BLA que exprimem CD103 difere em diversas destas patologias.[183, 187, 230]

Na população de sarcoidose que avaliamos (estudo IV) verificamos no BAL uma baixa expressão do $CD103^+$ na subpopulação $CD4^+$ e não na $CD8^+$. Subsequentemente, encontramos uma razão $CD103^+CD4^+/CD4^+$ no BAL significativamente mais baixa nesta patologia sendo que, independentemente da razão $CD4^+/CD8^+$, um *cutoff* de 0.25 na razão $CD103^+CD4^+/CD4^+$ apresentou uma elevada especificidade (91%) para o diagnóstico de sarcoidose.

A nossa investigação (estudo V) também demonstrou que, em contraste, os doentes com HP apresentaram contagens de linfócitos $CD103^+$ no BAL significativamente mais elevadas, em ambas as subpopulações $CD4^+$ e $CD8^+$, em comparação com a sarcoidose ou outras doenças intersticiais, como a IPF ou as associadas a doenças do tecido conetivo. Uma vez que a HP é uma doença granulomatosa pulmonar como a sarcoidose, por vezes com apresentação semelhante, e que as razões $CD4^+/CD8^+$ no BAL destes doentes podem ser altamente variáveis, novos marcadores fenotípicos com potencial de aumentarem a precisão do diagnóstico diferencial das alveolites linfocíticas podem ter também implicações práticas.

Como outros estudos têm sugerido, estes dados corroboram o conceito de que a diferente expressão fenotípica do CD103 nos linfócitos alveolares traduza a distinta imunopatologia que estará na base das diferenças clínicas destas duas doenças granulomatosas pulmonares.[183, 230] De fato, a relativa ausência do CD103 nos linfócitos $CD4^+$ na sarcoidose pulmonar sugere a origem periférica dessas células, suportando a hipótese de uma redistribuição do sangue periférico e a sua compartimentalização para o pulmão, já sugerida noutros estudos.[184, 187-189] Por outro lado, na HP, a expansão local, bronco-alveolar, de linfócitos intra-epiteliais ($CD103^+$) está de acordo com a sua expressão respiratória e um contato antigénico persistente, também por via inalatória.[184, 188] Adicionalmente, sabe-se que a subpopulação $CD4^+CD103^+$ se encontra sobretudo no compartimento de linfócitos T memória e que têm alguma capacidade reguladora, suprimindo a proliferação T, e que após ativação, esta subpopulação pode produzir IL-13, citocina que tem propriedades pró-fibróticas mediando a indução de fibrose pelo TGF-beta.[185, 186]

Tendo em conta estas observações experimentais, é possível que o predomínio de linfócitos CD103+ que observamos na HP possa refletir uma fase de reparação (i.e. supressão e/ou fibrose) da inflamação intersticial granulomatosa.

METALOPROTEINASES MMP1 E MMP7 NAS PNEUMONIAS INTERSTICIAIS FIBROSANTES E SARCOIDOISE PULMONAR (ESTUDO VI)

Neste grupo de doenças e nos estudos previamente publicados destacam-se a MMP-1 e principalmente a MMP-7, que pela sua expressão e propriedades têm sido associadas a pneumonias intersticiais fibrosantes, nomeadamente à IPF.[193, 194, 196, 231, 232] A sua expressão, habitualmente modesta em condições fisiológicas, aumenta de forma significativa nos processos de reparação.[194] Efetivamente, as MMPs estão envolvidas não só na degradação de componentes da matriz extra-celular, mas igualmente na modulação de uma variedade de mediadores, como citocinas e quimiocinas.[193, 194] As MMPs são reguladas a nível trancripcional e pós-transcripcional por inibidores e promotores sob condições fisiológicas, aumentando num processo de reparação, sendo que uma atividade descontrolada das MMPs resulta numa agressão tecidular com perda de função.[193, 194] A MMP-7, que aparenta ser a mais relevante em termos de expressão, nomeadamente na IPF, tem propriedades pró-fibróticas, com intervenção em vários processos biológicos, desde a resposta imunitária e inflamatória, apoptose e fibroproliferação.[196, 233-236] Estas observações permitem considerar estas MMP como relevantes no mecanismo fisiopatológico da fibrose, podendo inclusive, em caso da sua diferente expressão e participação nas diferentes patologias intersticiais fibrosantes, ter um papel no diagnóstico diferencial das mesmas.[194, 196] No contexto do estímulo e ativação de mediadores inflamatórios e pró-fibróticos, destaca-se a acção de libertação do TGF-beta da matriz extracelular, um dos passos fundamentais na regulação da sua atividade, sendo a TGF-beta um dos mediadores principais do desenvolvimento de fibrose.[12, 194] De acordo com o referido, verifica-se a presença de MMP-7 em células epiteliais e macrófagos alveolares de doentes com IPF, assim como nas células musculares lisas dos vasos, nos focos fibróticos e áreas de epitélio hiperplástico, mas não em indivíduos sem doença respiratória.[236, 237] Por outro lado, foi já demonstrada uma correlação significativa dos níveis séricos de MMP-7 com o agravamento funcional respiratório, tendo também sido descritos como marcadores de prognóstico na IPF, dada a associação independente com a mortalidade.[235, 237]

A MMP-1 tem igualmente várias funções biológicas como são o processamento de citocinas ou migração celular.[193, 235] Tem sido descrita o aumento da sua expressão pulmonar na IPF, embora curiosamente a nível do epitélio alveolar, mas não nos focos fibroblásticos ou no colagénio a nível intersticial, sendo sugerido que pode contribuir para a formação de espaços císticos no pulmão.[193, 235] O nosso estudo é o primeiro a relatar

um aumento significativo de MMP-1 sérico na IPF, comparativamente à UIP não-IPF.

Dado o padrão UIP não ser patognomónico, a IPF é sempre um diagnóstico de exclusão, tendo que ser investigadas eventuais doenças do tecido conjuntivo (CTD-ILD), HP ou toxicidade pulmonar induzida por drogas.[11, 12, 25] Por outro lado, nos casos de ausência de características imagiológicas típicas de UIP na HRCT (ex. UIP possível), pode tornar-se muito difícil a distinção entre a IPF e entidades como NSIP idiopática ou secundária.[11, 12, 25] O diagnóstico preciso de IPF é de importância fundamental, considerando as implicações terapêuticas e de prognóstico, sendo a biopsia pulmonar cirúrgica necessária em cerca de 1/3 dos casos.[11, 25] A biopsia pulmonar cirúrgica, no entanto, é um procedimento invasivo, com riscos associados (ex: de exacerbação aguda da IPF), e além disso, não é viável em doentes com doença grave ou com co-morbilidades relevantes.[238, 239] Seria de grande benefício a existência de biomarcadores de diagnóstico fiáveis. Baseado nos dados disponíveis, a MMP-1 e MMP-7 são as proteínas mais significativamente expressas no pulmão de doentes com IPF, em comparação com controlos saudáveis.[194] Têm sido descritas comparações com outras patologias, como a HP, embora maioritariamente formas subagudas, e sarcoidose (estado I e II), faltando no entanto a comparação com as formas que mais se assemelham com a IPF, e cuja diferenciação é mais difícil, como são os casos de UIP secundária nomeadamente no contexto de HP ou a NSIP de tipo fibrótico.[196] Esse foi um dos resultados do nosso estudo, em que verificamos, nomeadamente, que quer a MMP-1 quer, mais significativamente, a MMP-7 apresentaram diferenças significativas nos seus níveis séricos na IPF comparativamente com a UIP secundária, revelando assim o seu potencial como marcador para o diagnóstico diferencial.

IMPLICAÇÕES PRÁTICAS E INVESTIGAÇÃO FUTURA

Nos diferentes estudos efetuados, foram evidenciados alguns resultados que poderão ter potenciais implicações práticas na avaliação clínica das ILDs. No entanto, permanecem em aberto algumas questões, para além de outras interrogações que entretanto foram surgindo após a discussão dos resultados obtidos, e que poderão constituir a base da prossecução desta linha de investigação:

Baseados nos nossos resultados, em conjunto com outras séries com resultados e conclusões idênticas, fica evidente a associação do SNP *BTNL2 rs2076530* A com a sarcoidose em indivíduos caucasianos, podendo ser considerado um marcador de suscetibilidade da doença. Tendo em conta o desequilíbrio de ligação do *BTNL2* e HLA-*DR*, também demonstramos que o alelo A do *BTNL2 rs2076530* é fator de risco independente para a apresentação clínica da sarcoidose como doença pulmonar isolada. Por outro lado, não fica esclarecido o eventual papel como fator prognóstico deste SNP, pese embora dados existentes na literatura sugiram uma

associação com as formas mais graves da doença. Em primeiro lugar, é ainda necessária uma correta e rigorosa estratificação das várias formas de evolução na sarcoidose, não existindo até ao momento um consenso, o que certamente dificultará a identificação de fatores de prognóstico, nomeadamente genéticos. Os dados de *Wijnen et al.*, com uma larga amostragem populacional, são particularmente relevantes, dado que apontam para uma associação significativa com um subgrupo particular de doentes, aqueles com doença persistente, isto é a forma mais grave de evolução, com necessidade de intervenção terapêutica e maior instabilidade clínica. Estes dados são francamente promissores e deverão estimular estudos adicionais, eventualmente multicêntricos, de forma a conterem uma amostragem representativa de doentes com sarcoidose com diferentes tipos de evolução.

O polimorfismo *rs1049550**T (R230C *variant*) da anexina 11 tem sido descrito como tendo um efeito protetor relativamente à ocorrência da sarcoidose, independentemente da origem geográfica das populações estudadas, englobando até ao momento doentes caucasianos, asiáticos e afro-americanos, o que permite poder ser considerado um dos de maior fiabilidade entre os marcadores genéticos de suscetibilidade já descritos. Apesar de termos encontrado um aumento da frequência do genótipo TT da anexina A11 em doentes com a neutrofilia do LBA, não encontramos nenhuma associação com a regressão ou com a evolução crónica (mesmo considerando formas crónicas estáveis e persistentes) e igualmente tendo em conta 2 e 5 anos de evolução. No entanto, escasseiam dados acerca de uma eventual associação a fenotipos de apresentação clínica e principalmente à evolução, havendo necessidade de prosseguir a avaliação de eventuais associações deste SNP com formas de evolução devidamente estratificadas e definidas de forma rigorosa. Dado o papel da anexina A11 na divisão celular, apoptose e função neutrofílica, este polimorfismo poderá relacionar-se com a inflamação granulomatosa e intersticial que caracteriza a sarcoidose, uma hipótese a merecer também estudos futuros.

As principais características da sarcoidose, desde a suscetibilidade, à forma de apresentação clínica e evolução, são certamente determinados por uma interação de vários genes, o que poderá explicar em parte, a dificuldade de obtenção de um marcador genético de evolução isolado. Baseados nesta hipótese, não encontramos nenhuma interação significativa entre os alelos HLA e os SNPs estudados e apenas o HLA-*DRB1*03* se manteve associado à resolução da doença, apresentando-se assim como um fator de prognóstico clínico favorável, o que permitirá uma abordagem terapêutica mais assente na vigilância clínica.

A observação de um nível significativamente menor de células $CD4^+CD103^+$ no BAL dos doentes com sarcoidose, poderá ser um parâmetro útil no diagnóstico, independentemente da razão CD4/CD8. O nosso estudo revelou o valor de 0,45 como o ponto de corte da relação $CD4^+CD103/CD4^+$ no BAL com melhor relação diagnóstica (sensibilidade: 81%, especificidade: 78%), aumentando a especificidade com a diminuição do valor desta

relação (ex: 0,25-91%). Entretanto, a descrição por alguns autores de valores mais elevados das células $CD4^{+}CD103^{+}$ nos estadios mais graves da doença, deverá ser confirmada em estudos futuros, tendo em vista um potencial marcador de prognóstico que poderá vir a ser atribuído a esta subpopulação no BAL.

Embora tenhamos observado uma maior expressão de células $CD103^{+}$ no BAL dos doentes com doença dos criadores de aves, a diferença para a suberose não foi significativa, podendo este resultado relacionar-se com uma amostragem limitada. Será então necessária a investigação desta hipótese em grupos de maior dimensão de doentes com HP secundária a diferentes exposições. O diferente tipo de exposição antigénica nos doentes com HP, exemplificada no estudo por nós efetuada, com uma exposição intermitente na doença dos criadores de aves e mais persistente em doentes com exposição laboral, como a suberose, poderá acompanhar-se de uma diferente expressão do CD103 nos linfócitos da superfície bronco-alveolar.

Demonstrámos que os níveis séricos de MMP-1 e, principalmente, de MMP-7 são significativamente maiores na IPF que nas outras formas de UIP, configurando um elevado potencial de diagnóstico diferencial entre ILDs com alterações clínicas e imagiológicas que frequentemente se sobrepõem. A MMP-7 sérica inferior a 3,91 ng/ml apresentou um elevado valor preditivo negativo, de 82,4%, o que fornece um elemento adicional na exclusão de IPF na abordagem diagnóstica multidisciplinar destas entidades. Por outro lado, apesar de no nosso estudo a combinação das duas MMPs não trazer aparente benefício diagnóstico, estão descritos na literatura outros biomarcadores séricos que a eles associados ganham maior acuidade diagnóstica. Esta será uma via de investigação incontornável, partindo da relevância diagnóstica que estas MMP mostram, adicionadas a outros mediadores da IPF, que poderão aumentar ainda mais a precisão diagnóstica e prognóstica de biomarcadores séricos na IPF.

Conclusões

Nesta dissertação pretendemos investigar e analisar algumas das principais influências imunogenéticas e imunopatogénicas na suscetibilidade, expressão clínica e evolução das principais doenças do interstício pulmonar. A partir dos resultados obtidos e apresentados, muitos deles interrelacionados, podemos obter as seguintes conclusões:

1. Num grupo de doentes originários da população do Norte de Portugal verificamos um aumento significativo da frequência do alelo A do *BTNL2 rs2076530* em comparação com um grupo controlo, sendo a associação do genótipo AA com a doença ainda maior. Estes resultados corroboram outros estudos populacionais que associam este polimorfismo do *BTNL2*, um membro da superfamília das imunoglobulinas localizado na junção das regiões da classe II e classe III do HLA, com a suscetibilidade à sarcoidose.

2. Tendo em conta o desequilíbrio de ligação entre o *BTNL2* e o HLA-*DR*, também foi possível identificar dois fatores de risco independentes para diferentes apresentações clínicas da sarcoidose: o alelo A do *BTNL2 rs2076530*, em doentes sem sindroma de *Löfgren* ou com doença pulmonar isolada, e o alelo HLA-*DRB1*03* nos que apresentam sindroma de *Löfgren* ou resolução da doença nos 2 anos de seguimento após o diagnóstico.

3. Verificamos uma associação estatisticamente significativa com efeito protetor do SNP *rs1049550* C/T da anexina A11 com a susceptibilidade à sarcoidose na população portuguesa, com a particularidade do efeito protetor aumentar com o número de cópias no genótipo (um efeito gene-dose). No entanto, no nosso estudo o efeito protetor do alelo T não é estatisticamente significativo quando comparadas as suas frequências entre doentes com a síndroma de *Löfgren* e o grupo de controlos.

4. Apesar de termos encontrado um aumento da frequência do genótipo TT da anexina A11 em doentes com a neutrofilia do BAL, não obtivemos nenhuma associação estatisticamente significativa deste genótipo com a regressão ou com a evolução crónica (mesmo considerando formas crónicas estáveis e persistentes) e igualmente tendo em conta 2 e 5 anos de evolução. Dado o papel da anexina A11 na divisão celular, apoptose e função neutrofílica, este polimorfismo poderá influenciar elementos chave da inflamação granulomatosa e intersticial que carateriza a sarcoidose, uma hipótese a merecer estudos futuros.

5. Dado que a sarcoidose é uma doença multifatorial, investigamos possíveis interações gene-gene entre os alelos das classes I e II do HLA, o *BTNL2 rs2076530* G/A e o *ANXA 11 rs2076530* G/A, não se encontrando nenhuma interação estatisticamente significativa entre os alelos HLA e os SNPs estudados, nos modelos de regressão logística aplicados. Apenas para o *DRB1*03* se encontrou uma associação estatisticamente significatica com a regressão da doença, pelo que genes ligados à suscetibilidade não parecem associar-se, necessariamente, à evolução clínica da sarcoidose.

6. A expressão do CD103 nos linfócitos $CD4^+$ do BAL revela-se um parâmetro útil do diagnóstico de sarcoidose, independentemente da razão CD4/CD8, suportando a relevância deste parâmetro na fenotipagem linfocitária das doenças do interstício pulmonar. No nosso estudo podemos redefinir uma razão $CD103+CD4^+/CD4^+$ nos linfócitos do BAL ente 0,25 e 0,45, como um bom marcador diagnóstico da sarcoidose.

7. As Pneumonites de Hipersensibilidade apresentam valores significativamente mais elevados de linfócitos $CD103^+$ no BAL, quer na subpopulação $CD4^+$ quer $CD8^+$, corroborando a hipótese da linfocitose alveolar resultar, nesta patologia, de uma expansão local de linfócitos intra-epiteliais e das mucosas. Além disso, o estudo da expressão do CD103 é um elemento auxiliar na interpretação dos achados do LBA nas doenças do interstício pulmonar.

8. Avaliando os níveis séricos de MMP-1 e MMP-7, duas metaloproteinases que participam na remodelação da matriz extracelular e angiogénese, confirmamos o seu valor potencial como biomarcadores diagnósticos da Fibrose Pulmonar Idiopática (IPF). Uma vez que as UIP não-IPF colocam as maiores dificuldades no diagnóstico diferencial, os níveis significativamente mais elevados de MMP-1 e, em particular, MMP-7 na IPF poderão ser tidos em conta na abordagem diagnóstica multidisciplinar destas entidades.

Bibliografia

1. Behr J. *Approach to the diagnosis of interstitial lung disease. Clinics in chest medicine*. 2012 Mar;33(1):1-10. PubMed PMID: 22365241. Epub 2012/03/01.

2. Ryu JH, Daniels CE, Hartman TE, Yi ES. *Diagnosis of interstitial lung diseases.* Mayo Clinic proceedings. 2007 Aug;82(8):976-86. PubMed PMID: 17673067. Epub 2007/08/04.

3. Alhamad EH, Cosgrove GP. *Interstitial lung disease: the initial approach.* The Medical clinics of North America. 2011 Nov;95(6):1071-93. PubMed PMID: 22032428. Epub 2011/10/29.

4. Meyer KC. *Diagnosis and management of interstitial lung disease.* Translational respiratory medicine. 2014;2:4. PubMed PMID: 25505696. Pubmed Central PMCID: PMC4215823. Epub 2014/12/17.

5. Wallis A, Spinks K. *The diagnosis and management of interstitial lung diseases.* BMJ (Clinical research ed). 2015;350:h2072. PubMed PMID: 25952322. Epub 2015/05/09.

6. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. American journal of respiratory and critical care medicine. 2002 Jan 15;165(2):277-304. PubMed PMID: 11790668. Epub 2002/01/16.

7. Fischer A, Antoniou KM, Brown KK, Cadranel J, Corte TJ, du Bois RM, *et al. An official European Respiratory Society/American Thoracic Society research statement: interstitial pneumonia with autoimmune features.* The European respiratory journal. 2015 Jul 9. Pub Med PMID: 26160873. Epub 2015/07/15.

8. Fischer A, du Bois R. *Interstitial lung disease in connective tissue disorders.* Lancet (London, England). 2012 Aug 18;380(9842):689-98. PubMed PMID: 22901890. Epub 2012/08/21.

9. Tsuchiya Y, Fischer A, Solomon JJ, Lynch DA. *Connective Tissue Disease-related Thoracic Disease.* Clinics in chest medicine. 2015 Jun;36(2):283-97. PubMed PMID: 26024605. Epub 2015/05/31.

10. Travis WD, Costabel U, Hansell DM, King TE, Jr., Lynch DA, Nicholson AG, *et al. An official American Thoracic Society/European Respiratory Society statement: Update of the international multidisciplinary classification of the idiopathic interstitial pneumonias.* American journal of respiratory and critical care medicine. 2013 Sep 15;188(6):733-48. PubMed PMID: 24032382. Epub 2013/09/17.

11. Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, *et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for*

diagnosis and management. American journal of respiratory and critical care medicine. 2011 Mar 15;183(6):788-824. PubMed PMID: 21471066. Epub 2011/04/08.

12. King TE, Jr., Pardo A, Selman M. *Idiopathic pulmonary fibrosis*. Lancet (London, England). 2011 Dec 3;378(9807):1949-61. PubMed PMID: 21719092. Epub 2011/07/02.

13. Selman M, King TE, Pardo A. *Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy*. Annals of internal medicine. 2001 Jan 16;134(2):136-51. PubMed PMID: 11177318. Epub 2001/02/15.

14. Renzoni E, Srihari V, Sestini P. *Pathogenesis of idiopathic pulmonary fibrosis: review of recent findings.* F1000prime reports. 2014;6:69. PubMed PMID: 25165568. Pubmed Central PMCID: PMC4126534. Epub 2014/08/29.

15. Spagnolo P, Wells AU, Collard HR. *Pharmacological treatment of idiopathic pulmonary fibrosis: an update.* Drug discovery today. 2015 May;20(5):514-24. PubMed PMID: 25613790. Epub 2015/01/24.

16. Cottin V, Crestani B, Valeyre D, Wallaert B, Cadranel J, Dalphin JC, *et al*. *Diagnosis and management of idiopathic pulmonary fibrosis: French practical guidelines*. European respiratory review: an official journal of the European Respiratory Society. 2014 Jun;23(132):193-214. PubMed PMID: 24881074. Epub 2014/06/02.

17. Spagnolo P, Sverzellati N, Rossi G, Cavazza A, Tzouvelekis A, Crestani B, *et al*. *Idiopathic pulmonary fibrosis: an update*. Annals of medicine. 2015 Feb;47(1):15-27. PubMed PMID: 25613170. Epub 2015/01/24.

18. Ryu JH, Moua T, Daniels CE, Hartman TE, Yi ES, Utz JP, *et al*. I*diopathic pulmonary fibrosis: evolving concepts*. Mayo Clinic proceedings. 2014 Aug;89(8):1130-42. PubMed PMID: 24867394. Epub 2014/05/29.

19. Soares Pires F, Caetano Mota P, Melo N, Costa D, Jesus JM, Cunha R, *et al*. *Idiopathic pulmonary fibrosis--clinical presentation, outcome and baseline prognostic factors in a Portuguese cohort*. Revista portuguesa de pneumologia. 2013 Jan-Feb;19(1):19-27. PubMed PMID: 22819487. Epub 2012/07/24.

20. Katzenstein AL, Fiorelli RF. *Nonspecific interstitial pneumonia/fibrosis. Histologic features and clinical significance.* The American journal of surgical pathology. 1994 Feb; 18(2):136-47. PubMed PMID: 8291652. Epub 1994/02/01.

21. Travis WD, Hunninghake G, King TE, Jr., Lynch DA, Colby TV, Galvin JR, *et al*. *Idiopathic nonspecific interstitial pneumonia: report of an American Thoracic Society project*. American journal of respiratory and critical care medicine. 2008 Jun 15;177(12):1338-47. PubMed PMID: 18388353. Epub 2008/04/05.

22. Poletti V, Romagnoli M, Piciucchi S, Chilosi M. *Current status of idiopathic nonspecific interstitial pneumonia.* Seminars in respiratory and critical care medicine. 2012 Oct;33(5): 440-9. PubMed PMID: 23001799. Epub 2012/09/25.

23. Kinder BW, Collard HR, Koth L, Daikh DI, Wolters PJ, Elicker B, *et al*. *Idiopathic nonspecific interstitial pneumonia: lung manifestation of undifferentiated connective tissue disease?* American journal of respiratory and critical care medicine. 2007 Oct 1;176(7):691-7. PubMed PMID: 17556720. Pubmed Central PMCID: PMC1994238. Epub 2007/06/09.

24. Nunes H, Schubel K, Piver D, Magois E, Feuillet S, Uzunhan Y, *et al*. *Nonspecific interstitial pneumonia: survival is influenced by the underlying cause*. The European respiratory journal. 2015 Mar;45(3):746-55. PubMed PMID: 25537566. Epub 2014/12/30.

25. Bradley B, Branley HM, Egan JJ, Greaves MS, Hansell DM, Harrison NK, *et al*. *Interstitial lung disease guideline: the British Thoracic Society in collaboration with the Thoracic Society of Australia and New Zealand and the Irish Thoracic Society.* Thorax. 2008 Sep;63 Suppl 5:v1-58. PubMed PMID: 18757459. Epub 2008/09/11.

26. Margaritopoulos GA, Harari S, Caminati A, Antoniou KM. *Smoking-related idiopathic interstitial pneumonia: A review.* Respirology (Carlton, Vic). 2015 Jul 2. PubMed PMID: 26138798. Epub 2015/07/04.

27. Caminati A, Cavazza A, Sverzellati N, Harari S. *An integrated approach in the diagnosis of smoking-related interstitial lung diseases.* European respiratory review: an official journal of the European Respiratory Society. 2012 Sep 1;21(125):207-17. PubMed PMID: 22941885. Epub 2012/09/04.

28. Davies G, Wells AU, du Bois RM. *Respiratory bronchiolitis associated with interstitial lung disease and desquamative interstitial pneumonia.* Clinics in chest medicine. 2004 Dec; 25(4):717-26, vi. PubMed PMID: 15564017. Epub 2004/11/27.

29. Vaz M, Melo N, Mota PC, Cunha R, Pereira JM. *Evaluation of clinical presentation and outcome of patients with respiratory bronchiolitis-associated interstitial lung disease.* Rev Port Pneumol (2006). 2015 Jan-Feb;21(1):47-8. PubMed PMID: 25854138. Epub 2015/04/10.

30. Nair A, Hansell DM. High-resolution computed tomography features of smoking-related interstitial lung disease. Seminars in ultrasound, CT, and MR. 2014 Feb;35(1):59-71. PubMed PMID: 24480144. Epub 2014/02/01.

31. Baloira A, Xaubet A, Rodriguez Becerra E, Romero AD, Casanova A, Ancochea J. [Desquamative interstitial pneumonia and respiratory bronchiolitis-associated interstitial lung disease: data from the Spanish patient registry]. Arch Bronconeumol. 2008 Sep;44(9):499-503. PubMed PMID: 19000514. Epub 2008/11/13. Neumonia intersticial descamativa y bronquiolitis respiratoria asociada a enfermedad pulmonar intersticial: datos del registro espanol.

32. Ryu JH, Myers JL, Capizzi SA, Douglas WW, Vassallo R, Decker PA. Desquamative interstitial pneumonia and respiratory bronchiolitis-associated interstitial lung disease. Chest. 2005 Jan;127(1):178-84. PubMed PMID: 15653981. Epub 2005/01/18.

33. Cordier JF. Cryptogenic organising pneumonia. The European respiratory journal. 2006 Aug;28(2):422-46. PubMed PMID: 16880372. Epub 2006/08/02.

34. Cottin V, Cordier JF. Cryptogenic organizing pneumonia. Seminars in respiratory and critical care medicine. 2012 Oct;33(5):462-75. PubMed PMID: 23001801. Epub 2012/09/25.

35. Lazor R. [Cryptogenic and secondary organizing pneumonia]. La Revue du praticien. 2007 Dec 31;57(20):2243-8. PubMed PMID: 18320744. Epub 2008/03/07. Pneumopathie organisee cryptogenique ou secondaire.

36. Lazor R, Vandevenne A, Pelletier A, Leclerc P, Court-Fortune I, Cordier JF. *Cryptogenic organizing pneumonia. Characteristics of relapses in a series of 48 patients.* The Groupe d'Etudes et de Recherche sur les Maladles "Orphelines" Pulmonaires (GERM"O"P). American journal of respiratory and critical care medicine. 2000 Aug;162(2 Pt 1):571-7. PubMed PMID: 10934089. Epub 2000/08/10.

37. Frankel SK, Cool CD, Lynch DA, Brown KK. *Idiopathic pleuroparenchymal fibroelastosis: description of a novel clinicopathologic entity*. Chest. 2004 Dec;126(6):2007-13. PubMed PMID: 15596706. Epub 2004/12/15.

38. Redondo MT, Melo N, Mota PC, Jesus JM, Moura CS, Guimaraes S, *et al*. *Idiopathic pleuroparenchymal fibroelastosis: a rare but increasingly recognized entity.* Rev Port Pneumol (2006). 2015 Jan-Feb;21(1):41-4. PubMed PMID: 25854135. Epub 2015/04/10.

39. Oda T, Ogura T, Kitamura H, Hagiwara E, Baba T, Enomoto Y, *et al. Distinct characteristics of pleuroparenchymal fibroelastosis with usual interstitial pneumonia compared with idiopathic pulmonary fibrosis.* Chest. 2014 Nov;146(5):1248-55. PubMed PMID: 25367470. Epub 2014/11/05.

40. Piciucchi S, Tomassetti S, Casoni G, Sverzellati N, Carloni A, Dubini A, *et al. High resolution CT and histological findings in idiopathic pleuroparenchymal fibroelastosis: features and differential diagnosis.* Respiratory research. 2011;12:111. PubMed PMID: 21861891. Pubmed Central PMCID: PMC3197499. Epub 2011/08/25.

41. Kusagaya H, Nakamura Y, Kono M, Kaida Y, Kuroishi S, Enomoto N, *et al. Idiopathic pleuroparenchymal fibroelastosis: consideration of a clinicopathological entity in a series of Japanese patients.* BMC pulmonary medicine. 2012;12:72. PubMed PMID: 23216996. Pubmed Central PMCID: PMC3539991. Epub 2012/12/12.

42. Camus P, von der Thusen J, Hansell DM, Colby TV. *Pleuroparenchymal fibroelastosis: one more walk on the wild side of drugs?* The European respiratory journal. 2014 Aug;44 (2):289-96. PubMed PMID: 25082907. Epub 2014/08/02.

43. Skolnik K, Ryerson CJ. *Unclassifiable interstitial lung disease: A review.* Respirology (Carlton, Vic). 2015 Jun 9. PubMed PMID: 26059704. Epub 2015/06/11.

44. Ryerson CJ, Urbania TH, Richeldi L, Mooney JJ, Lee JS, Jones KD, *et al. Prevalence and prognosis of unclassifiable interstitial lung disease.* The European respiratory journal. 2013 Sep;42(3):750-7. PubMed PMID: 23222877. Epub 2012/12/12.

45. *Statement on sarcoidosis.* Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. American journal of respiratory and critical care medicine. 1999 Aug;160(2):736-55. PubMed PMID: 10430755. Epub 1999/08/03.

46. Valeyre D, Prasse A, Nunes H, Uzunhan Y, Brillet PY, Muller-Quernheim J. *Sarcoidosis.* Lancet (London, England). 2014 Mar 29;383(9923):1155-67. PubMed PMID: 24090799. Epub 2013/10/05.

47. Culver DA. *Diagnosing sarcoidosis*. Current opinion in pulmonary medicine. 2015 Jul 14. PubMed PMID: 26176972. Epub 2015/07/16.

48. Nunes H, Soler P, Valeyre D. *Pulmonary sarcoidosis.* Allergy. 2005 May;60(5):565-82. PubMed PMID: 15813801. Epub 2005/04/09.

49. Baughman RP, Culver DA, Judson MA. *A concise review of pulmonary sarcoidosis.* American journal of respiratory and critical care medicine. 2011 Mar 1;183(5):573-81. PubMed PMID: 21037016. Pubmed Central PMCID: PMC3081278. Epub 2010/11/03.

50. Rybicki BA, Iannuzzi MC. *Epidemiology of sarcoidosis: recent advances and future prospects.* Seminars in respiratory and critical care medicine. 2007 Feb;28(1):22-35. PubMed PMID: 17330190. Epub 2007/03/03.

51. Newman LS, Rose CS, Bresnitz EA, Rossman MD, Barnard J, Frederick M, *et al. A case control etiologic study of sarcoidosis: environmental and occupational risk factors.*

American journal of respiratory and critical care medicine. 2004 Dec 15;170(12):1324-30. PubMed PMID: 15347561. Epub 2004/09/07.

52. Iannuzzi MC, Rybicki BA, Teirstein AS. *Sarcoidosis.* The New England journal of medicine. 2007 Nov 22;357(21):2153-65. PubMed PMID: 18032765. Epub 2007/11/23.

53. Judson MA. *The Clinical Features of Sarcoidosis: A Comprehensive Review.* Clinical reviews in allergy & immunology. 2014 Oct 2. PubMed PMID: 25274450. Epub 2014/10/03.

54. Nunes H, Uzunhan Y, Freynet O, Naccache JM, Bouvry D, Brillet PY, *et al. [Thoracic sarcoidosis].* Presse Med. 2010 Jan;39(1):93-106. PubMed PMID: 19959325. Epub 2009/12/05. Sarcoidose mediastinopulmonaire.

55. Valeyre D, Bernaudin JF, Uzunhan Y, Kambouchner M, Brillet PY, Soussan M, *et al. Clinical presentation of sarcoidosis and diagnostic work-up*. Seminars in respiratory and critical care medicine. 2014 Jun;35(3):336-51. PubMed PMID: 25007086. Epub 2014/07/10.

56. Tejera Segura B, Holgado S, Mateo L, Pego-Reigosa JM, Carnicero Iglesias M, Olive A. *[Lofgren syndrome: a study of 80 cases].* Medicina clinica. 2014 Aug 19;143(4):166-9. PubMed PMID: 24855899. Epub 2014/05/27. Sindrome de Lofgren: estudio de 80 casos.

57. Scadding JG. *Prognosis of intrathoracic sarcoidosis in England. A review of 136 cases after five years' observation.* British medical journal. 1961 Nov 4;2(5261):1165-72. PubMed PMID: 14497750. Pubmed Central PMCID: PMC1970202. Epub 1961/11/04.

58. Nunes H, Brillet PY, Valeyre D, Brauner MW, Wells AU. *Imaging in sarcoidosis.* Seminars in respiratory and critical care medicine. 2007 Feb;28(1):102-20. PubMed PMID: 17330195. Epub 2007/03/03.

59. Lynch JP, 3rd. Computed tomographic scanning in sarcoidosis. Seminars in respiratory and critical care medicine. 2003 Aug;24(4):393-418. PubMed PMID: 16088560. Epub 2005/08/10.

60. Silva M, Nunes H, Valeyre D, Sverzellati N. Imaging of Sarcoidosis. Clinical reviews in allergy & immunology. 2015 Aug;49(1):45-53. PubMed PMID: 25726413. Epub 2015/03/03.

61. Spagnolo P, Sverzellati N, Wells AU, Hansell DM. Imaging aspects of the diagnosis of sarcoidosis. European radiology. 2014 Apr;24(4):807-16. PubMed PMID: 24399491. Epub 2014/01/09.

62. Meyer KC, Raghu G, Baughman RP, Brown KK, Costabel U, du Bois RM, *et al. An official American Thoracic Society clinical practice guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease*. American journal of respiratory and critical care medicine. 2012 May 1;185(9):1004-14. PubMed PMID: 22550210. Epub 2012/05/03.

63. Costabel U, Guzman J. *Bronchoalveolar lavage in interstitial lung disease.* Current opinion in pulmonary medicine. 2001 Sep;7(5):255-61. PubMed PMID: 11584173. Epub 2001/ 10/05.

64. Drent M, Mansour K, Linssen C. *Bronchoalveolar lavage in sarcoidosis*. Seminars in respiratory and critical care medicine. 2007 Oct;28(5):486-95. PubMed PMID: 17975776. Epub 2007/11/03.

65. Goyal A, Gupta D, Agarwal R, Bal A, Nijhawan R, Aggarwal AN. *Value of different bronchoscopic sampling techniques in diagnosis of sarcoidosis: a prospective study of 151 patients.* Journal of bronchology & interventional pulmonology. 2014 Jul;21(3):220-6. PubMed PMID: 24992130. Epub 2014/07/06.

66. Lynch JP, 3rd, Ma YL, Koss MN, White ES. *Pulmonary sarcoidosis*. Seminars in respiratory and critical care medicine. 2007 Feb;28(1):53-74. PubMed PMID: 17330192. Epub 2007/03/03.

67. Mukhopadhyay S, Gal AA. *Granulomatous lung disease: an approach to the differential diagnosis*. Archives of pathology & laboratory medicine. 2010 May;134(5):667-90. PubMed PMID: 20441499. Epub 2010/05/06.

68. Culver DA, Costabel U. *EBUS-TBNA for the diagnosis of sarcoidosis: is it the only game in town?* Journal of bronchology & interventional pulmonology. 2013 Jul;20(3):195-7. Pub Med PMID: 23857191. Epub 2013/07/17.

69. Narula T, Baughman RP, Mehta AC. "Sarcoidosis Americana-route Europa". Journal of bronchology & interventional pulmonology. 2013 Oct;20(4):293-6. PubMed PMID: 24162109. Epub 2013/10/29.

70. von Bartheld MB, Dekkers OM, Szlubowski A, Eberhardt R, Herth FJ, in 't Veen JC, *et al. Endosonography vs conventional bronchoscopy for the diagnosis of sarcoidosis: the GRANULOMA randomized clinical trial.* Jama. 2013 Jun 19;309(23):2457-64. PubMed PMID: 23780458. Epub 2013/06/20.

71. Israel-Biet D, Valeyre D. *Diagnosis of pulmonary sarcoidosis.* Current opinion in pulmonary medicine. 2013 Sep;19(5):510-5. PubMed PMID: 23880701. Epub 2013/07/25.

72. Nunes H, Bouvry D, Soler P, Valeyre D. *Sarcoidosis*. Orphanet journal of rare diseases. 2007;2:46. PubMed PMID: 18021432. Pubmed Central PMCID: Pmc2169207. Epub 2007/11/21.

73. Judson MA. *Extrapulmonary sarcoidosis.* Seminars in respiratory and critical care medicine. 2007 Feb;28(1):83-101. PubMed PMID: 17330194. Epub 2007/03/03.

74. Judson MA, Costabel U, Drent M, Wells A, Maier L, Koth L, *et al. The WASOG Sarcoidosis Organ Assessment Instrument: An update of a previous clinical tool.* Sarcoidosis, vasculitis, and diffuse lung diseases : official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders. 2014;31(1):19-27. PubMed PMID: 24751450. Epub 2014/04/23.

75. Judson MA, Baughman RP. *Worsening of pulmonary sarcoidosis.* Current opinion in pulmonary medicine. 2014 Sep;20(5):508-16. PubMed PMID: 25022316. Epub 2014/07/16.

76. Baughman RP, Lower EE. *Medical therapy of sarcoidosis*. Seminars in respiratory and critical care medicine. 2014 Jun;35(3):391-406. PubMed PMID: 25007090. Epub 2014/07/10.

77. Nardi A, Brillet PY, Letoumelin P, Girard F, Brauner M, Uzunhan Y, *et al. Stage IV sarcoidosis: comparison of survival with the general population and causes of death*. The European respiratory journal. 2011 Dec;38(6):1368-73. PubMed PMID: 22075486. Epub 2011/11/15.

78. Baughman RP, Nunes H. *Therapy for sarcoidosis: evidence-based recommendations.* Expert review of clinical immunology. 2012 Jan;8(1):95-103. PubMed PMID: 22149344. Epub 2011/12/14.

79. Selman M, Pardo A, King TE, Jr. *Hypersensitivity pneumonitis: insights in diagnosis and pathobiology.* American journal of respiratory and critical care medicine. 2012 Aug 15;186(4):314-24. PubMed PMID: 22679012. Epub 2012/06/09.

80. Selman M, Buendia-Roldan I. *Immunopathology, diagnosis, and management of hypersensitivity pneumonitis.* Seminars in respiratory and critical care medicine. 2012 Oct;33 (5):543-54. PubMed PMID: 23001807. Epub 2012/09/25.

81. Lacasse Y, Cormier Y. *Hypersensitivity pneumonitis.* Orphanet journal of rare diseases. 2006;1:25. PubMed PMID: 16817954. Pubmed Central PMCID: PMC1533805. Epub 2006/07/05.

82. Lacasse Y, Girard M, Cormier Y. *Recent advances in hypersensitivity pneumonitis.* Chest. 2012 Jul;142(1):208-17. PubMed PMID: 22796841. Epub 2012/07/17.

83. Morais A, Winck JC, Delgado L, Palmares MC, Fonseca J, Moura e Sa J, *et al. Suberosis and bird fancier's disease: a comparative study of radiological, functional and bronchoalveolar lavage profiles.* Journal of investigational allergology & clinical immunology. 2004; 14(1):26-33. PubMed PMID: 15160439. Epub 2004/05/27.

84. Camarena A, Juarez A, Mejia M, Estrada A, Carrillo G, Falfan R, *et al. Major histocompatibility complex and tumor necrosis factor-alpha polymorphisms in pigeon breeder's disease*. American journal of respiratory and critical care medicine. 2001 Jun;163(7):1528-33. PubMed PMID: 11401868. Epub 2001/06/13.

85. Falfan-Valencia R, Camarena A, Pineda CL, Montano M, Juarez A, Buendia-Roldan I, *et al. Genetic susceptibility to multicase hypersensitivity pneumonitis is associated with the TNF-238 GG genotype of the promoter region and HLA-DRB1*04 bearing HLA haplotypes.* Respiratory medicine. 2014 Jan;108(1):211-7. PubMed PMID: 24291122. Epub 2013/12/03.

86. Selman M, Pardo A, Barrera L, Estrada A, Watson SR, Wilson K, *et al. Gene expression profiles distinguish idiopathic pulmonary fibrosis from hypersensitivity pneumonitis.* American journal of respiratory and critical care medicine. 2006 Jan 15;173(2):188-98. PubMed PMID: 16166619. Pubmed Central PMCID: PMC2662988. Epub 2005/09/17.

87. Aquino-Galvez A, Camarena A, Montano M, Juarez A, Zamora AC, Gonzalez-Avila G, *et al. Transporter associated with antigen processing (TAP) 1 gene polymorphisms in patients with hypersensitivity pneumonitis.* Experimental and molecular pathology. 2008 Apr;84(2):173-7. PubMed PMID: 18342853. Epub 2008/03/18.

88. Kondoh K, Usui Y, Ohtani Y, Inase N, Miyake S, Yoshizawa Y. *Proinflammatory and anti-inflammatory cytokine gene polymorphisms in hypersensitivity pneumonitis.* Journal of medical and dental sciences. 2006 Mar;53(1):75-83. PubMed PMID: 16722148. Epub 2006/05/26.

89. Girard M, Cormier Y. *Hypersensitivity pneumonitis.* Current opinion in allergy and clinical immunology. 2010 Apr;10(2):99-103. PubMed PMID: 20093932. Epub 2010/01/23.

90. Lacasse Y, Selman M, Costabel U, Dalphin JC, Morell F, Erkinjuntti-Pekkanen R, *et al. Classification of hypersensitivity pneumonitis: a hypothesis.* International archives of allergy and immunology. 2009;149(2):161-6. PubMed PMID: 19127074. Epub 2009/01/08.

91. Costabel U, Bonella F, Guzman J. *Chronic hypersensitivity pneumonitis*. Clinics in chest medicine. 2012 Mar;33(1):151-63. PubMed PMID: 22365252. Epub 2012/03/01.

92. Silva CI, Churg A, Muller NL. *Hypersensitivity pneumonitis: spectrum of high-resolution CT and pathologic findings*. AJR American journal of roentgenology. 2007 Feb;188(2):334-44. PubMed PMID: 17242239. Epub 2007/01/24.

93. Patel RA, Sellami D, Gotway MB, Golden JA, Webb WR. *Hypersensitivity pneumonitis: patterns on high-resolution CT.* Journal of computer assisted tomography. 2000 Nov-Dec ;24(6):965-70. PubMed PMID: 11105719. Epub 2000/12/06.

94. Silva CI, Muller NL, Lynch DA, Curran-Everett D, Brown KK, Lee KS, *et al. Chronic hypersensitivity pneumonitis: differentiation from idiopathic pulmonary fibrosis and*

nonspecific interstitial pneumonia by using thin-section CT. Radiology. 2008 Jan;246(1):288-97. PubMed PMID: 18096541. Epub 2007/12/22.

95. Morell F, Villar A, Montero MA, Munoz X, Colby TV, Pipvath S, *et al. Chronic hypersensitivity pneumonitis in patients diagnosed with idiopathic pulmonary fibrosis: a prospective case-cohort study.* The Lancet Respiratory medicine. 2013 Nov;1(9):685-94. PubMed PMID: 24429272. Epub 2014/01/17.

96. van den Bosch JM, Heye C, Wagenaar SS, van Velzen-Blad HC. *Bronchoalveolar lavage in extrinsic allergic alveolitis*. Respiration; international review of thoracic diseases. 1986;49(1):45-51. PubMed PMID: 3485301. Epub 1986/01/01.

97. Costabel U. *The alveolitis of hypersensitivity pneumonitis.* The European respiratory journal. 1988 Jan;1(1):5-9. PubMed PMID: 3284760. Epub 1988/01/01.

98. Ohshimo S, Bonella F, Cui A, Beume M, Kohno N, Guzman J, *et al. Significance of bronchoalveolar lavage for the diagnosis of idiopathic pulmonary fibrosis.* American journal of respiratory and critical care medicine. 2009 Jun 1;179(11):1043-7. PubMed PMID: 19246718. Epub 2009/02/28.

99. Gupta N, Vassallo R, Wikenheiser-Brokamp KA, McCormack FX. *Diffuse Cystic Lung Disease. Part I.* American journal of respiratory and critical care medicine. 2015 Jun 15;191(12):1354-66. PubMed PMID: 25906089. Epub 2015/04/24.

100. Gupta N, Vassallo R, Wikenheiser-Brokamp KA, McCormack FX. *Diffuse Cystic Lung Disease. Part II.* American journal of respiratory and critical care medicine. 2015 Jul 1;192(1):17-29. PubMed PMID: 25906201. Epub 2015/04/24.

101. Ryu JH, Tian X, Baqir M, Xu K. *Diffuse cystic lung diseases*. Frontiers of medicine. 2013 Sep;7(3):316-27. PubMed PMID: 23666611. Epub 2013/05/15.

102. Neel A, Artifoni M, Donadieu J, Lorillon G, Hamidou M, Tazi A. *[Langerhans cell histiocytosis in adults].* La Revue de medecine interne / fondee par la Societe nationale francaise de medecine interne. 2015 Jul 3. PubMed PMID: 26150351. Epub 2015/07/08. Histiocytose langerhansienne de l'adulte.

103. Suri HS, Yi ES, Nowakowski GS, Vassallo R. *Pulmonary langerhans cell histiocytosis.* Orphanet journal of rare diseases. 2012;7:16. PubMed PMID: 22429393. Pubmed Central PMCID: PMC3342091. Epub 2012/03/21.

104. Elia D, Torre O, Cassandro R, Caminati A, Harari S. *Pulmonary Langerhans cell histiocytosis: a comprehensive analysis of 40 patients and literature review*. European journal of internal medicine. 2015 Jun;26(5):351-6. PubMed PMID: 25899682. Epub 2015/04/23.

105. Tazi A, de Margerie C, Naccache JM, Fry S, Dominique S, Jouneau S, *et al. The natural history of adult pulmonary Langerhans cell histiocytosis: a prospective multicentre study.* Orphanet journal of rare diseases. 2015;10:30. PubMed PMID: 25887097. Pubmed Central PMCID: PMC4438520. Epub 2015/04/19.

106. Tazi A, Marc K, Dominique S, de Bazelaire C, Crestani B, Chinet T, *et al. Serial computed tomography and lung function testing in pulmonary Langerhans' cell histiocytosis.* The European respiratory journal. 2012 Oct;40(4):905-12. PubMed PMID: 22441752. Epub 2012/03/24.

107. Canuet M, Kessler R, Jeung MY, Metivier AC, Chaouat A, Weitzenblum E. *Correlation between high-resolution computed tomography findings and lung function in*

pulmonary Langerhans cell histiocytosis. Respiration; international review of thoracic diseases. 2007;74(6):640-6. PubMed PMID: 17675829. Epub 2007/08/07.

108. Taveira-DaSilva AM, Moss J. *Clinical features, epidemiology, and therapy of lymphangioleiomyomatosis.* Clinical epidemiology. 2015;7:249-57. PubMed PMID: 25897262. Pubmed Central PMCID: PMC4396456. Epub 2015/04/22.

109. Taveira-DaSilva AM, Moss J. *Management of lymphangioleiomyomatosis.* F1000prime reports. 2014;6:116. PubMed PMID: 25580270. Pubmed Central PMCID: PMC4251421. Epub 2015/01/13.

110. Henske EP, McCormack FX. *Lymphangioleiomyomatosis - a wolf in sheep's clothing.* The Journal of clinical investigation. 2012 Nov;122(11):3807-16. PubMed PMID: 23114603. Pubmed Central PMCID: PMC3484429. Epub 2012/11/02.

111. Paciocco G, Uslenghi E, Bianchi A, Mazzarella G, Roviaro GC, Vecchi G, *et al. Diffuse cystic lung diseases: correlation between radiologic and functional status.* Chest. 2004 Jan;125(1):135-42. PubMed PMID: 14718432. Epub 2004/01/14.

112. von Ranke FM, Zanetti G, JL ES, Neto CA, Godoy MC, Souza CA, *et al. Tuberous Sclerosis Complex: State-of-the-Art Review with a Focus on Pulmonary Involvement.* Lung. 2015 Jun 24. PubMed PMID: 26104489. Epub 2015/06/25.

113. Borie R, Danel C, Debray MP, Taille C, Dombret MC, Aubier M, *et al. Pulmonary alveolar proteinosis.* European respiratory review : an official journal of the European Respiratory Society. 2011 Jun;20(120):98-107. PubMed PMID: 21632797. Epub 2011/06/03.

114. Bonella F, Bauer PC, Griese M, Ohshimo S, Guzman J, Costabel U. *Pulmonary alveolar proteinosis: new insights from a single-center cohort of 70 patients*. Respiratory medicine. 2011 Dec;105(12):1908-16. PubMed PMID: 21900000. Epub 2011/09/09.

115. Campo I, Mariani F, Rodi G, Paracchini E, Tsana E, Piloni D, *et al. Assessment and management of pulmonary alveolar proteinosis in a reference center.* Orphanet journal of rare diseases. 2013;8:40. PubMed PMID: 23497546. Pubmed Central PMCID: PMC3605309. Epub 2013/03/19.

116. Chartrand S, Fischer A. *Management of connective tissue disease-associated interstitial lung disease.* Rheumatic diseases clinics of North America. 2015 May;41(2):279-94. PubMed PMID: 25836643. Epub 2015/04/04.

117. de Lauretis A, Veeraraghavan S, Renzoni E. *Review series: Aspects of interstitial lung disease: connective tissue disease-associated interstitial lung disease: how does it differ from IPF? How should the clinical approach differ?* Chronic respiratory disease. 2011;8(1):53-82. PubMed PMID: 21339375. Epub 2011/02/23.

118. Lally L, Spiera RF. *Pulmonary vasculitis.* Rheumatic diseases clinics of North America. 2015 May;41(2):315-31. PubMed PMID: 25836645. Epub 2015/04/04.

119. Yunt ZX, Frankel SK, Brown KK. *Diagnosis and management of pulmonary vasculitis.* Therapeutic advances in respiratory disease. 2012 Dec;6(6):375-90. PubMed PMID: 22878624. Epub 2012/08/11.

120. Wemeau-Stervinou L, Wallaert B. *[Diagnostic approach of interstitial lung diseases].* La Revue du praticien. 2014 Sep;64(7):957-62. PubMed PMID: 25362779. Epub 2014/11/05. Conduite diagnostique des pneumopathies interstitielles diffuses.

121. Costabel U, Guzman J, Bonella F, Oshimo S. *Bronchoalveolar lavage in other interstitial lung diseases.* Seminars in respiratory and critical care medicine. 2007 Oct;28(5):514-24. PubMed PMID: 17975779. Epub 2007/11/03.

122. Meyer KC. *The role of bronchoalveolar lavage in interstitial lung disease.* Clinics in chest medicine. 2004 Dec;25(4):637-49, v. PubMed PMID: 15564013. Epub 2004/11/27.

123. Meyer KC. *Bronchoalveolar lavage as a diagnostic tool.* Seminars in respiratory and critical care medicine. 2007 Oct;28(5):546-60. PubMed PMID: 17975782. Epub 2007/11/03.

124. Reynolds HY. *Present status of bronchoalveolar lavage in interstitial lung disease.* Current opinion in pulmonary medicine. 2009 Sep;15(5):479-85. PubMed PMID: 19636227. Epub 2009/07/29.

125. *Technical recommendations and guidelines for bronchoalveolar lavage (BAL)*. Report of the European Society of Pneumology Task Group. The European respiratory journal. 1989 Jun;2(6):561-85. PubMed PMID: 2663535. Epub 1989/06/01.

126. Israel-Biet D, Danel C. *[Contribution of bronchoalveolar lavage to the management of interstitial lung disease].* Revue de pneumologie clinique. 2005 Jun;61(3):141-8. PubMed PMID: 16142186. Epub 2005/09/06. Contribution du lavage bronchoalveolaire a la prise en charge des pneumopathies interstitielles diffuses.

127. Baughman RP. *Technical aspects of bronchoalveolar lavage: recommendations for a standard procedure*. Seminars in respiratory and critical care medicine. 2007 Oct;28(5):475-85. PubMed PMID: 17975775. Epub 2007/11/03.

128. Wells AU. *The clinical utility of bronchoalveolar lavage in diffuse parenchymal lung disease.* European respiratory review : an official journal of the European Respiratory Society. 2010 Sep;19(117):237-41. PubMed PMID: 20956199. Epub 2010/10/20.

129. Descombes E, Gardiol D, Leuenberger P. *Transbronchial lung biopsy: an analysis of 530 cases with reference to the number of samples.* Monaldi archives for chest disease = Archivio Monaldi per le malattie del torace / Fondazione clinica del lavoro, IRCCS [and] Istituto di clinica tisiologica e malattie apparato respiratorio, Universita di Napoli, Secondo ateneo. 1997 Aug;52(4):324-9. PubMed PMID: 9401359. Epub 1997/08/01.

130. Fraire AE, Cooper SP, Greenberg SD, Rowland LP, Langston C. *Transbronchial lung biopsy. Histopathologic and morphometric assessment of diagnostic utility.* Chest. 1992 Sep;102(3):748-52. PubMed PMID: 1516396. Epub 1992/09/01.

131. Morell F, Reyes L, Domenech G, De Gracia J, Majo J, Ferrer J. *[Diagnoses and diagnostic procedures in 500 consecutive patients with clinical suspicion of interstitial lung disease].* Arch Bronconeumol. 2008 Apr;44(4):185-91. PubMed PMID: 18423179. Epub 2008/04/22. Diagnosticos y procedimientos en 500 pacientes consecutivos con sospecha clinica de enfermedad pulmonar intersticial.

132. Valenti S, Scordamaglia A, Crimi P, Mereu C. *Bronchoalveolar lavage and transbronchial lung biopsy in sarcoidosis and extrinsic allergic alveolitis.* European journal of respiratory diseases. 1982 Nov;63(6):564-9. PubMed PMID: 7173351. Epub 1982/11/01.

133. Danel C. *[Usefulness of transbronchial and surgical biopsies for the management of interstitial lung disease].* Revue de pneumologie clinique. 2005 Jun;61(3):149-57. PubMed PMID: 16142187. Epub 2005/09/06. Interet des biopsies transbronchiques et chirurgicales dans la prise en charge des pneumopathies interstitielles diffuses.

134. Margaritopoulos GA, Wells AU. *The role of transbronchial biopsy in the diagnosis of diffuse parenchymal lung diseases: con.* Revista portuguesa de pneumologia. 2012 Mar-Apr;18(2):61-3. PubMed PMID: 22264995. Epub 2012/01/24.

135. Poletti V, Casoni GL, Gurioli C, Ryu JH, Tomassetti S. *Lung cryobiopsies: a paradigm shift in diagnostic bronchoscopy?* Respirology (Carlton, Vic). 2014 Jul;19(5):645-54. Pub Med PMID: 24862226. Epub 2014/05/28.

136. Casoni GL, Gurioli C, Chhajed PN, Chilosi M, Zompatori M, Olivieri D, *et al. The value of transbronchial lung biopsy using jumbo forceps via rigid bronchoscope in diffuse lung disease*. Monaldi archives for chest disease = Archivio Monaldi per le malattie del torace / Fondazione clinica del lavoro, IRCCS [and] Istituto di clinica tisiologica e malattie apparato respiratorio, Universita di Napoli, Secondo ateneo. 2008 Jun;69(2):59-64. PubMed PMID: 18837418. Epub 2008/10/08.

137. Casoni GL, Cordeiro CR, Jr., Tomassetti S, Romagnoli M, Chilosi M, Cancellieri A, *et al. The role of transbronchial biopsy in the diagnosis of diffuse parenchymal lung diseases: pro.* Revista portuguesa de pneumologia. 2012 Mar-Apr;18(2):57-60. PubMed PMID: 21719249. Epub 2011/07/02.

138. Babiak A, Hetzel J, Krishna G, Fritz P, Moeller P, Balli T, *et al. Transbronchial cryobiopsy: a new tool for lung biopsies.* Respiration; international review of thoracic diseases. 2009;78(2):203-8. PubMed PMID: 19246874. Epub 2009/02/28.

139. Pajares V, Torrego A, Puzo C, Lerma E, Gil De Bernabe MA, Franquet T. *[Transbronchial lung biopsy using cryoprobes].* Arch Bronconeumol. 2010 Mar;46(3):111-5. PubMed PMID: 19939546. Epub 2009/11/27. Utilizacion de criosondas para la realizacion de la biopsia pulmonar transbronquial.

140. Pajares V, Puzo C, Castillo D, Lerma E, Montero MA, Ramos-Barbon D, *et al. Diagnostic yield of transbronchial cryobiopsy in interstitial lung disease: a randomized trial.* Respirology (Carlton, Vic). 2014 Aug;19(6):900-6. PubMed PMID: 24890124. Epub 2014/06/04.

141. Casoni GL, Tomassetti S, Cavazza A, Colby TV, Dubini A, Ryu JH, *et al. Transbronchial lung cryobiopsy in the diagnosis of fibrotic interstitial lung diseases.* PloS one. 2014; 9(2):e86716. PubMed PMID: 24586252. Pubmed Central PMCID: PMC3938401. Epub 2014/ 03/04.

142. Hagmeyer L, Theegarten D, Wohlschlager J, Treml M, Matthes S, Priegnitz C, *et al. The role of transbronchial cryobiopsy and surgical lung biopsy in the diagnostic algorithm of interstitial lung disease.* The clinical respiratory journal. 2015 Jan 26. PubMed PMID: 25620578. Epub 2015/01/27.

143. Nguyen W, Meyer KC. *Surgical lung biopsy for the diagnosis of interstitial lung disease: a review of the literature and recommendations for optimizing safety and efficacy.* Sarcoidosis, vasculitis, and diffuse lung diseases: official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders. 2013 Mar;30(1):3-16. PubMed PMID: 24003530. Epub 2013/09/06.

144. Flaherty KR, Travis WD, Colby TV, Toews GB, Kazerooni EA, Gross BH, *et al. Histopathologic variability in usual and nonspecific interstitial pneumonias.* American journal of respiratory and critical care medicine. 2001 Nov 1;164(9):1722-7. PubMed PMID: 11719316. Epub 2001/11/24.

145. Fibla JJ, Molins L, Blanco A, Royo I, Martinez Vallina P, Martinez N, *et al. Video-assisted thoracoscopic lung biopsy in the diagnosis of interstitial lung disease: a prospective, multi-*

center study in 224 patients. Arch Bronconeumol. 2012 Mar;48(3):81-5. PubMed PMID: 22244947. Epub 2012/01/17.

146. Iannuzzi MC, Rybicki BA. *Genetics of sarcoidosis: candidate genes and genome scans.* Proceedings of the American Thoracic Society. 2007 Jan;4(1):108-16. PubMed PMID: 17202299. Pubmed Central PMCID: Pmc2647608. Epub 2007/01/05.

147. Fischer A, Grunewald J, Spagnolo P, Nebel A, Schreiber S, Muller-Quernheim J. *Genetics of sarcoidosis.* Seminars in respiratory and critical care medicine. 2014 Jun;35 (3):296-306. PubMed PMID: 25007082. Epub 2014/07/10.

148. Grunewald J. *Review: role of genetics in susceptibility and outcome of sarcoidosis.* Seminars in respiratory and critical care medicine. 2010 Aug;31(4):380-9. PubMed PMID: 20665388. Epub 2010/07/29.

149. Grunewald J, Brynedal B, Darlington P, Nisell M, Cederlund K, Hillert J, *et al. Different HLA-DRB1 allele distributions in distinct clinical subgroups of sarcoidosis patients.* Respiratory research. 2010;11:25. PubMed PMID: 20187937. Pubmed Central PMCID: PMC2846896. Epub 2010/03/02.

150. Grunewald J, Spagnolo P, Wahlstrom J, Eklund A. *Immunogenetics of Disease-Causing Inflammation in Sarcoidosis.* Clinical reviews in allergy & immunology. 2015 Aug;49(1):19-35. PubMed PMID: 25791751. Epub 2015/03/21.

151. Fischer A, Rybicki BA. *Granuloma genes in sarcoidosis: what is new?* Current opinion in pulmonary medicine. 2015 Jul 8. PubMed PMID: 26164297. Epub 2015/07/15.

152. Morais A, Alves H, Lima B, Delgado L, Goncalves R, Tafulo S. *HLA class I and II and TNF-alpha gene polymorphisms in sarcoidosis patients.* Revista portuguesa de pneumologia. 2008 Nov-Dec;14(6):727-46. PubMed PMID: 19023492. Epub 2008/11/22.

153. Iannuzzi MC, Fontana JR. *Sarcoidosis: clinical presentation, immunopathogenesis, and therapeutics.* Jama. 2011 Jan 26;305(4):391-9. PubMed PMID: 21266686. Epub 2011/01/27.

154. Ziegenhagen MW, Muller-Quernheim J. *The cytokine network in sarcoidosis and its clinical relevance.* Journal of internal medicine. 2003 Jan;253(1):18-30. PubMed PMID: 12588535. Epub 2003/02/18. .

155. Zissel G, Prasse A, Muller-Quernheim J. *Immunologic response of sarcoidosis.* Seminars in respiratory and critical care medicine. 2010 Aug;31(4):390-403. PubMed PMID: 20665389. Epub 2010/07/29.

156. Zissel G. *Cellular activation in the immune response of sarcoidosis.* Seminars in respiratory and critical care medicine. 2014 Jun;35(3):307-15. PubMed PMID: 25007083. Epub 2014/07/10.

157. Agostini C, Facco M, Chilosi M, Semenzato G. *Alveolar macrophage-T cell interactions during Th1-type sarcoid inflammation.* Microscopy research and technique. 2001 May 15;53(4):278-87. PubMed PMID: 11340673. Epub 2001/05/08.

158. Xie HJ, Wu M, Niu Y, Shen B, Huo Y, Cheng Y. *Associations between tumor necrosis factor alpha gene polymorphism and sarcoidosis: a meta-analysis.* Molecular biology reports. 2014 Jul;41(7):4475-80. PubMed PMID: 24604725. Epub 2014/03/08.

159. Kuroda H, Saijo Y, Fujiuchi S, Takeda H, Ohsaki Y, Hasebe N. *Relationship between cytokine single nucleotide polymorphisms and sarcoidosis among Japanese subjects.* Sarcoidosis, vasculitis, and diffuse lung diseases : official journal of WASOG / World

Association of Sarcoidosis and Other Granulomatous Disorders. 2013 Mar;30(1):36-42. Pub Med PMID: 24003533. Epub 2013/09/06.

160. Vasakova M, Sterclova M, Kolesar L, Slavcev A, Skibova J, Striz I. *Cytokine gene polymorphisms in sarcoidosis.* Sarcoidosis, vasculitis, and diffuse lung diseases : official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders. 2010 Jul;27(1):70-5. PubMed PMID: 21086908. Epub 2010/11/23.

161. Spagnolo P, Grunewald J. *Recent advances in the genetics of sarcoidosis.* Journal of medical genetics. 2013 May;50(5):290-7. PubMed PMID: 23526832. Epub 2013/03/26.

162. Spagnolo P, du Bois RM. *Genetics of sarcoidosis.* Clinics in dermatology. 2007 May-Jun;25(3):242-9. PubMed PMID: 17560301. Epub 2007/06/15.

163. Arnett HA, Escobar SS, Viney JL. *Regulation of costimulation in the era of butyrophilins.* Cytokine. 2009 Jun;46(3):370-5. PubMed PMID: 19380239. Epub 2009/04/22.

164. Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, *et al. BTNL2, a butyrophilin/B7-like molecule, is a negative costimulatory molecule modulated in intestinal inflammation.* Journal of immunology (Baltimore, Md : 1950). 2007 Feb 1;178 (3):1523-33. PubMed PMID: 17237401. Epub 2007/01/24.

165. Nguyen T, Liu XK, Zhang Y, Dong C. *BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation.* Journal of immunology (Baltimore, Md : 1950). 2006 Jun 15;176(12):7354-60. PubMed PMID: 16751379. Pubmed Central PMCID: PMC1626526. Epub 2006/06/06.

166. Rybicki BA, Walewski JL, Maliarik MJ, Kian H, Iannuzzi MC. *The BTNL2 gene and sarcoidosis susceptibility in African Americans and Whites.* American journal of human genetics. 2005 Sep;77(3):491-9. PubMed PMID: 16080124. Pubmed Central PMCID: PMC 1226214. Epub 2005/08/05.

167. Spagnolo P, Sato H, Grutters JC, Renzoni EA, Marshall SE, Ruven HJ, *et al. Analysis of BTNL2 genetic polymorphisms in British and Dutch patients with sarcoidosis.* Tissue antigens. 2007 Sep;70(3):219-27. PubMed PMID: 17661910. Epub 2007/07/31.

168. Valentonyte R, Hampe J, Huse K, Rosenstiel P, Albrecht M, Stenzel A, et al. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. Nature genetics. 2005 Apr;37(4):357-64. PubMed PMID: 15735647. Epub 2005/03/01.

169. Li Y, Wollnik B, Pabst S, Lennarz M, Rohmann E, Gillissen A, *et al. BTNL2 gene variant and sarcoidosis.* Thorax. 2006 Mar;61(3):273-4. PubMed PMID: 16517590. Pubmed Central PMCID: PMC2080740. Epub 2006/03/07.

170. Wijnen PA, Voorter CE, Nelemans PJ, Verschakelen JA, Bekers O, Drent M. *Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression?* Human immunology. 2011 Apr;72(4):342-7. PubMed PMID: 21256912. Epub 2011/01/25.

171. Hofmann S, Franke A, Fischer A, Jacobs G, Nothnagel M, Gaede KI, *et al. Genome-wide association study identifies ANXA11 as a new susceptibility locus for sarcoidosis*. Nature genetics. 2008 Sep;40(9):1103-6. PubMed PMID: 19165924. Epub 2009/01/24.

172. Mrazek F, Stahelova A, Kriegova E, Fillerova R, Zurkova M, Kolek V, *et al. Functional variant ANXA11 R230C: true marker of protection and candidate disease modifier in sarcoidosis.* Genes and immunity. 2011 Sep;12(6):490-4. PubMed PMID: 21562576. Epub 2011/05/13.

173. Levin AM, Iannuzzi MC, Montgomery CG, Trudeau S, Datta I, McKeigue P, *et al. Association of ANXA11 genetic variation with sarcoidosis in African Americans and European Americans.* Genes and immunity. 2013 Jan;14(1):13-8. PubMed PMID: 23151485. Pubmed Central PMCID: PMC3595044. Epub 2012/11/16.

174. Li Y, Pabst S, Kubisch C, Grohe C, Wollnik B. *First independent replication study confirms the strong genetic association of ANXA11 with sarcoidosis.* Thorax. 2010 Oct;65(10):939-40. PubMed PMID: 20805159. Epub 2010/09/02.

175. Fatimathas L, Moss SE. *Characterisation of the sarcoidosis-associated variant of annexin A11.* General physiology and biophysics. 2009;28 Spec No Focus:F29-38. PubMed PMID: 20093723. Epub 2010/01/23.

176. Fatimathas L, Moss SE. *Annexins as disease modifiers.* Histology and histopathology. 2010 Apr;25(4):527-32. PubMed PMID: 20183805. Epub 2010/02/26.

177. Tomas A, Futter C, Moss SE. *Annexin 11 is required for midbody formation and completion of the terminal phase of cytokinesis.* The Journal of cell biology. 2004 Jun 21;165(6):813-22. PubMed PMID: 15197175. Pubmed Central PMCID: PMC2172404. Epub 2004/06/16.

178. Vito P, Lacana E, D'Adamio L. *Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3.* Science (New York, NY). 1996 Jan 26;271(5248):521-5. PubMed PMID: 8560270. Epub 1996/01/26.

179. Heron M, Grutters JC, Van Moorsel CH, Ruven HJ, Kazemier KM, Claessen AM, *et al. Effect of variation in ITGAE on risk of sarcoidosis, CD103 expression, and chest radiography.* Clinical immunology (Orlando, Fla). 2009 Oct;133(1):117-25. PubMed PMID: 19604725. Epub 2009/07/17.

180. Kilshaw PJ. *Alpha E beta 7.* Molecular pathology: MP. 1999 Aug;52(4):203-7. PubMed PMID: 10694940. Pubmed Central PMCID: PMC395700. Epub 2000/03/01.

181. Erle DJ, Pabst R. *Intraepithelial lymphocytes in the lung: a neglected lymphocyte population.* American journal of respiratory cell and molecular biology. 2000 Apr;22(4):398-400. PubMed PMID: 10745019. Epub 2000/04/04.

182. Braun RK, Foerster M, Grahmann PR, Haefner D, Workalemahu G, Kroegel C. *Phenotypic and molecular characterization of CD103+ CD4+ T cells in bronchoalveolar lavage from patients with interstitial lung diseases.* Cytometry Part B, Clinical cytometry. 2003 Jul;54(1):19-27. PubMed PMID: 12827664. Epub 2003/06/27.

183. Lohmeyer J, Friedrich J, Grimminger F, Maus U, Tenter R, Morr H, *et al. Expression of mucosa-related integrin alphaEbeta7 on alveolar T cells in interstitial lung diseases.* Clinical and experimental immunology. 1999 May;116(2):340-6. PubMed PMID: 10337028. Pubmed Central PMCID: PMC1905287. Epub 1999/05/26.

184. Heron M, Slieker WA, Zanen P, van Lochem EG, Hooijkaas H, van den Bosch JM, *et al. Evaluation of CD103 as a cellular marker for the diagnosis of pulmonary sarcoidosis.* Clinical immunology (Orlando, Fla). 2008 Mar;126(3):338-44. PubMed PMID: 18182176. Epub 2008/01/10.

185. Lehmann J, Huehn J, de la Rosa M, Maszyna F, Kretschmer U, Krenn V, *et al. Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25 - regulatory T cells.* Proceedings of the National Academy of Sciences of the United States of America. 2002 Oct 1;99(20):13031-6. PubMed PMID: 12242333. Pubmed Central PMCID: PMC130581. Epub 2002/09/21.

186. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. *IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis.* Nature medicine. 2006 Jan;12(1):99-106. PubMed PMID: 16327802. Epub 2005/12/06.

187. Kolopp-Sarda MN, Kohler C, De March AK, Bene MC, Faure G. *Discriminative immunophenotype of bronchoalveolar lavage CD4 lymphocytes in sarcoidosis*. Laboratory investigation; a journal of technical methods and pathology. 2000 Jul;80(7):1065-9. PubMed PMID: 10908151. Epub 2000/07/25.

188. Semenzato G. *Immunology of interstitial lung diseases: cellular events taking place in the lung of sarcoidosis, hypersensitivity pneumonitis and HIV infection.* The European respiratory journal. 1991 Jan;4(1):94-102. PubMed PMID: 2026243. Epub 1991/01/01.

189. Chailleux E, Bignon JD, Peyrat MA, Godard A, Soulillou JP. *Lymphocyte subsets, phytohaemagglutinin responsiveness of blood lymphocytes, and interleukin 2 production in sarcoidosis.* Thorax. 1985 Oct;40(10):768-73. PubMed PMID: 3877349. Pubmed Central PMCID: PMC460181. Epub 1985/10/01.

190. Gaxiola M, Buendia-Roldan I, Mejia M, Carrillo G, Estrada A, Navarro MC, *et al. Morphologic diversity of chronic pigeon breeder's disease: clinical features and survival.* Respiratory medicine. 2011 Apr;105(4):608-14. PubMed PMID: 21167698. Epub 2010/12/21.

191. Nunes H, Maurer C, Naccache JM, Girard F, Brauner M, Valeyre D. *[Severe pulmonary sarcoidosis].* Annales de medecine interne. 2001 Mar;152(2):96-102. PubMed PMID: 11357045. Epub 2001/05/18. Sarcoidose pulmonaire grave.

192. Wuyts WA, Agostini C, Antoniou KM, Bouros D, Chambers RC, Cottin V, *et al. The pathogenesis of pulmonary fibrosis: a moving target.* The European respiratory journal. 2013 May;41(5):1207-18. PubMed PMID: 23100500. Epub 2012/10/27.

193. Dancer RC, Wood AM, Thickett DR. *Metalloproteinases in idiopathic pulmonary fibrosis.* The European respiratory journal. 2011 Dec;38(6):1461-7. PubMed PMID: 21700608. Epub 2011/06/28.

194. Pardo A, Selman M. *Role of matrix metaloproteases in idiopathic pulmonary fibrosis.* Fibrogenesis & tissue repair. 2012;5(Suppl 1):S9. PubMed PMID: 23259796. Pubmed Central PMCID: PMC3368759. Epub 2012/12/25.

195. Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, *et al. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans.* Proceedings of the National Academy of Sciences of the United States of America. 2002 Apr 30;99(9):6292-7. PubMed PMID: 11983918. Pubmed Central PMCID: PMC122942. Epub 2002/05/02.

196. Rosas IO, Richards TJ, Konishi K, Zhang Y, Gibson K, Lokshin AE, *et al. MMP1 and MMP7 as potential peripheral blood biomarkers in idiopathic pulmonary fibrosis*. PLoS medicine. 2008 Apr 29;5(4):e93. PubMed PMID: 18447576. Pubmed Central PMCID: PMC2346504. Epub 2008/05/02.

197. Schuyler M, Cormier Y. *The diagnosis of hypersensitivity pneumonitis.* Chest. 1997 Mar; 111(3):534-6. PubMed PMID: 9118683. Epub 1997/03/01.

198. Antoniou KM, Margaritopoulos GA, Tomassetti S, Bonella F, Costabel U, Poletti V. *Interstitial lung disease.* European respiratory review: an official journal of the European Respiratory Society. 2014 Mar 1;23(131):40-54. PubMed PMID: 24591661. Epub 2014/03/05.

199. Bonniaud P, Georges M, Favrolt N, Camus P. *[Drug-induced interstitial lung diseases].* La Revue du praticien. 2014 Sep;64(7):951-6. PubMed PMID: 25362778. Epub 2014/11/05. Pneumopathies interstitielles iatrogeniques.

200. Camus P, Bonniaud P, Fanton A, Camus C, Baudaun N, Foucher P. *Drug-induced and iatrogenic infiltrative lung disease.* Clinics in chest medicine. 2004 Sep;25(3):479-519, vi. PubMed PMID: 15331188. Epub 2004/08/28.

201. Fernandez Alvarez R, Martinez Gonzalez C, Quero Martinez A, Blanco Perez JJ, Carazo Fernandez L, Prieto Fernandez A. *Guidelines for the diagnosis and monitoring of silicosis.* Arch Bronconeumol. 2015 Feb;51(2):86-93. PubMed PMID: 25479706. Epub 2014/12/07.

202. Seaman DM, Meyer CA, Kanne JP. *Occupational and Environmental Lung Disease.* Clinics in chest medicine. 2015 Jun;36(2):249-68. PubMed PMID: 26024603. Epub 2015/05/31.

203. Judson MA, Baughman RP, Thompson BW, Teirstein AS, Terrin ML, Rossman MD, *et al. Two year prognosis of sarcoidosis: the ACCESS experience.* Sarcoidosis, vasculitis, and diffuse lung diseases: official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders. 2003 Oct;20(3):204-11. PubMed PMID: 14620163. Epub 2003/11/19.

204. Excoffier L, Laval G, Schneider S. *Arlequin (version 3.0): an integrated software package for population genetics data analysis.* Evolutionary bioinformatics online. 2005;1:47-50. PubMed PMID: 19325852. Pubmed Central PMCID: PMC2658868. Epub 2005/01/01.

205. Guo SW, Thompson EA. *Performing the exact test of Hardy-Weinberg proportion for multiple alleles.* Biometrics. 1992 Jun;48(2):361-72. PubMed PMID: 1637966. Epub 1992/06/01.

206. Wennerstrom A, Pietinalho A, Vauhkonen H, Lahtela L, Palikhe A, Hedman J, *et al. HLA-DRB1 allele frequencies and C4 copy number variation in Finnish sarcoidosis patients and associations with disease prognosis.* Human immunology. 2012 Jan;73(1):93-100. PubMed PMID: 22074998. Epub 2011/11/15.

207. Hedfors E, Lindstrom F. HLA-B8/DR3 in sarcoidosis. *Correlation to acute onset disease with arthritis.* Tissue antigens. 1983 Sep;22(3):200-3. PubMed PMID: 6636112. Epub 1983/09/01.

208. Gardner J, Kennedy HG, Hamblin A, Jones E. *HLA associations in sarcoidosis: a study of two ethnic groups.* Thorax. 1984 Jan;39(1):19-22. PubMed PMID: 6582657. Pubmed Central PMCID: PMC459715. Epub 1984/01/01.

209. Sato H, Woodhead FA, Ahmad T, Grutters JC, Spagnolo P, van den Bosch JM, *et al. Sarcoidosis HLA class II genotyping distinguishes differences of clinical phenotype across ethnic groups.* Human molecular genetics. 2010 Oct 15;19(20):4100-11. PubMed PMID: 20685690. Pubmed Central PMCID: PMC4351376. Epub 2010/08/06.

210. Berlin M, Fogdell-Hahn A, Olerup O, Eklund A, Grunewald J. *HLA-DR predicts the prognosis in Scandinavian patients with pulmonary sarcoidosis.* American journal of respiratory and critical care medicine. 1997 Nov;156(5):1601-5. PubMed PMID: 9372682. Epub 1997/12/31.

211. Iannuzzi MC, Maliarik MJ, Poisson LM, Rybicki BA. *Sarcoidosis susceptibility and resistance HLA-DQB1 alleles in African Americans.* American journal of respiratory and critical care medicine. 2003 May 1;167(9):1225-31. PubMed PMID: 12615619. Epub 2003/03/05.

212. Sato H, Grutters JC, Pantelidis P, Mizzon AN, Ahmad T, Van Houte AJ, *et al. HLA-DQB1*0201: a marker for good prognosis in British and Dutch patients with sarcoidosis.* American journal of respiratory cell and molecular biology. 2002 Oct;27(4):406-12. PubMed PMID: 12356573. Epub 2002/10/03.

213. Voorter CE, Drent M, van den Berg-Loonen EM. *Severe pulmonary sarcoidosis is strongly associated with the haplotype HLA-DQB1*0602-DRB1*150101.* Human immunology. 2005 Jul;66(7):826-35. PubMed PMID: 16112030. Epub 2005/08/23.

214. Wennerstrom A, Pietinalho A, Lasota J, Salli K, Surakka I, Seppanen M, *et al. Major histocompatibility complex class II and BTNL2 associations in sarcoidosis.* The European respiratory journal. 2013 Aug;42(2):550-3. PubMed PMID: 23904553. Epub 2013/08/02.

215. Bogunia-Kubik K, Tomeczko J, Suchnicki K, Lange A. *HLA-DRB1*03, DRB1*11 or DRB1*12 and their respective DRB3 specificities in clinical variants of sarcoidosis.* Tissue antigens. 2001 Jan;57(1):87-90. PubMed PMID: 11169267. Epub 2001/02/13.

216. Milman N, Svendsen CB, Nielsen FC, van Overeem Hansen T. *The BTNL2 A allele variant is frequent in Danish patients with sarcoidosis.* The clinical respiratory journal. 2011 Apr; 5(2):105-11. PubMed PMID: 21410903. Epub 2011/03/18.

217. Ozdemir M, Saydam F, Kurt E, Degirmenci I, Tuncel T, Cilingir O, *et al. Is there a genetic predisposition for Turkish patients with sarcoidosis in the 329-bp region containing the BTNL2 rs2076530 polymorphism?* Turkish journal of medical sciences. 2014;44(4):590-4. PubMed PMID: 25551927. Epub 2015/01/02.

218. Suzuki H, Ota M, Meguro A, Katsuyama Y, Kawagoe T, Ishihara M, *et al. Genetic characterization and susceptibility for sarcoidosis in Japanese patients: risk factors of BTNL2 gene polymorphisms and HLA class II alleles.* Investigative ophthalmology & visual science. 2012 Oct;53(11):7109-15. PubMed PMID: 22991420. Epub 2012/09/20.

219. Gaillot-Drevon M, Calender A, Blay JY, Valeyre D, Israel-Biet D, Roy P, *et al. Lack of correlation of BTNL2 polymorphism and cancer risk in sarcoidosis. BTNL2 and cancer risk in sarcoidosis.* Sarcoidosis, vasculitis, and diffuse lung diseases: official journal of WASOG/ World Association of Sarcoidosis and Other Granulomatous Disorders. 2014;31(2):136-41. PubMed PMID: 25078641. Epub 2014/08/01.

220. Coudurier M, Freymond N, Aissaoui S, Calender A, Pacheco Y, Devouassoux G. *Homozygous variant rs2076530 of BTNL2 and familial sarcoidosis.* Sarcoidosis, vasculitis, and diffuse lung diseases: official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders. 2009 Jul;26(2):162-6. PubMed PMID: 20560297. Epub 2010/06/22.

221. Bargagli E, Bianchi N, Margollicci M, Olivieri C, Luddi A, Coviello G, *et al. Chitotriosidase and soluble IL-2 receptor: comparison of two markers of sarcoidosis severity.* Scandinavian journal of clinical and laboratory investigation. 2008;68(6):479-83. PubMed PMID: 18609101. Epub 2008/07/09.

222. Facco M, Baesso I, Miorin M, Bortoli M, Cabrelle A, Boscaro E, *et al. Expression and role of CCR6/CCL20 chemokine axis in pulmonary sarcoidosis.* Journal of leukocyte biology. 2007 Oct;82(4):946-55. PubMed PMID: 17615381. Epub 2007/07/07.

223. Lin Y, Wei J, Fan L, Cheng D. *BTNL2 gene polymorphism and sarcoidosis susceptibility: a meta-analysis.* PloS one. 2015;10(4):e0122639. PubMed PMID: 25849037. Pubmed Central PMCID: PMC4388687. Epub 2015/04/08.

224. Baughman RP, Nagai S, Balter M, Costabel U, Drent M, du Bois R, *et al. Defining the clinical outcome status (COS) in sarcoidosis: results of WASOG Task Force.* Sarcoidosis, vasculitis, and diffuse lung diseases: official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders. 2011 Jul;28(1):56-64. PubMed PMID: 21796892. Epub 2011/07/30.

225. Mizutani A, Usuda N, Tokumitsu H, Minami H, Yasui K, Kobayashi R, *et al. CAP-50, a newly identified annexin, localizes in nuclei of cultured fibroblast 3Y1 cells.* The Journal of biological chemistry. 1992 Jul 5;267(19):13498-504. PubMed PMID: 1618851. Epub 1992/07/05.

226. Burke DF, Worth CL, Priego EM, Cheng T, Smink LJ, Todd JA, *et al. Genome bioinformatic analysis of nonsynonymous SNPs.* BMC bioinformatics. 2007;8:301. PubMed PMID: 17708757. Pubmed Central PMCID: PMC1978506. Epub 2007/08/22.

227. Kuriyama R, Gustus C, Terada Y, Uetake Y, Matuliene J. *CHO1, a mammalian kinesin-like protein, interacts with F-actin and is involved in the terminal phase of cytokinesis.* The Journal of cell biology. 2002 Mar 4;156(5):783-90. PubMed PMID: 11877456. Pubmed Central PMCID: PMC2173305. Epub 2002/03/06.

228. Feng X, Zang S, Yang Y, Zhao S, Li Y, Gao X, *et al. Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population: a case-control study.* BMJ open. 2014;4(7):e004466. PubMed PMID: 25056970. Pubmed Central PMCID: PMC4120255. Epub 2014/07/25.

229. Kantrow SP, Meyer KC, Kidd P, Raghu G. *The CD4/CD8 ratio in BAL fluid is highly variable in sarcoidosis.* The European respiratory journal. 1997 Dec;10(12):2716-21. PubMed PMID: 9493649. Epub 1998/03/11.

230. Rihs S, Walker C, Virchow JC, Jr., Boer C, Kroegel C, Giri SN, *et al. Differential expression of alpha E beta 7 integrins on bronchoalveolar lavage T lymphocyte subsets: regulation by alpha 4 beta 1-integrin crosslinking and TGF-beta.* American journal ofrespiratory cell and molecular biology. 1996 Nov;15(5):600-10. PubMed PMID: 8918367. Epub 1996/11/01.

231. McKeown S, Richter AG, O'Kane C, McAuley DF, Thickett DR. *MMP expression and abnormal lung permeability are important determinants of outcome in IPF.* The European respiratory journal. 2009 Jan;33(1):77-84. PubMed PMID: 18829682. Epub 2008/10/03.

232. Suga M, Iyonaga K, Okamoto T, Gushima Y, Miyakawa H, Akaike T, American journal of respiratory and critical care medicine. 2000 Nov;162(5):1949-56. PubMed PMID: 11069839. Epub 2000/11/09.

233. Putman RK, Rosas IO, Hunninghake GM. *Genetics and early detection in idiopathic pulmonary fibrosis.* American journal of respiratory and critical care medicine. 2014 Apr 1;189(7):770-8. PubMed PMID: 24547893. Pubmed Central PMCID: PMC4225831. Epub 2014/02/20.

234. Richards TJ, Kaminski N, Baribaud F, Flavin S, Brodmerkel C, Horowitz D, *et al. Peripheral blood proteins predict mortality in idiopathic pulmonary fibrosis.* American journal of respiratory and critical care medicine. 2012 Jan 1;185(1):67-76. PubMed PMID: 22016448. Pubmed Central PMCID: PMC3262037. Epub 2011/10/22.

235. Pardo A, Selman M, Kaminski N. *Approaching the degradome in idiopathic pulmonary fibrosis.* The international journal of biochemistry & cell biology. 2008;40(6-7):1141-55. PubMed PMID: 18207447. Epub 2008/01/22.

236. Fujishima S, Shiomi T, Yamashita S, Yogo Y, Nakano Y, Inoue T, *et al. Production and activation of matrix metalloproteinase 7 (matrilysin 1) in the lungs of patients with idiopathic pulmonary fibrosis.* Archives of pathology & laboratory medicine. 2010 Aug;134(8):1136-42. PubMed PMID: 20670133. Epub 2010/07/31.

237. Song JW, Do KH, Jang SJ, Colby TV, Han S, Kim DS. *Blood biomarkers MMP-7 and SP-A: predictors of outcome in idiopathic pulmonary fibrosis*. Chest. 2013 May;143(5):1422-9. PubMed PMID: 23715088. Epub 2013/05/30.

238. Blanco M, Obeso GA, Duran JC, Rivo JE, Garcia-Fontan E, Pena E, *et al. Surgical lung biopsy for diffuse lung disease. Our experience in the last 15 years.* Revista portuguesa de pneumologia. 2013 Mar-Apr;19(2):59-64. PubMed PMID: 23395290. Epub 2013/02/12.

239. Kaarteenaho R. T*he current position of surgical lung biopsy in the diagnosis of idiopathic pulmonary fibrosis.* Respiratory research. 2013;14:43. PubMed PMID: 23587070. Pubmed Central PMCID: PMC3639087. Epub 2013/04/17.

Agradecimentos

Ao Sr. Professor Doutor Agostinho Marques, orientador desta tese e diretor do serviço de Pneumologia do Centro Hospitalar São João, onde me formei e trabalho, pela referência e exemplo maior ao longo destes anos, para além do estímulo permanente.

Ao Sr. Professor Doutor Luis Delgado, co-orientador desta tese, pelo exemplo de rigor e profundidade científica, decisivos na realização de todo o trabalho de investigação e publicação. Agradeço igualmente a dedicação, estímulo, labor e amizade permanentes, sem as quais não teria sido possível concretizar todo o trabalho apresentado.

A todos os meus companheiros do grupo de Doenças Pulmonares Difusas, Dra. Natália Melo e Dra. Patrícia Mota, pela colaboração, lealdade e solidariedade inexcedíveis, aos Dr. José Miguel Pereira e Dr. Rui Cunha e às Dra. Conceição Souto Moura e Dra. Susana Guimarães pela colaboração incansável e de excelência, para além da disponibilidade permanente. Em conjunto, conseguimos criar um grupo de colaboração harmoniosa que tem permitido uma assistência de qualidade crescente a um número significativo de doentes. Gostaria de estender os agradecimentos a todos os que tem colaborado com a consulta de grupo de doenças pulmonares difusas, nomeadamente à Dra. Carla Damas que na consulta de transplante pulmonar tem prestado uma colaboração inestímável assim como à Dra. Adriana Magalhães em nome da qual quero agradecer toda a colaboração prestada por todos os componentes da equipa responsável pela realização das técnicas endoscópicas.

A todo o grupo do serviço de Imunologia do Centro Hospitalar São João, responsável pelo processamento do Lavado Broncoalveolar, à Dra. Carmo Palmares por longos anos de intensa colaboração, à Dra. Marília Beltrão, à Dra. Oksana Sokhatska e mais uma vez ao Prof. Luís Delgado.

A todos os meus colegas do serviço de Pneumologia do Centro Hospitalar São João, sem exceção, que ao longo de todos estes anos prestaram uma colaboração permanente ao grupo de doenças pulmonares difusas, contribuindo assim de forma decisiva para o seu desenvolvimento.

Ao Dr. Bruno Lima e à Dra. Helena Alves que no Instituto de Histocompatibilidade do Norte foram responsáveis pela orientação da investigação genética laboratorial das amostras dos doentes com sarcoidose, pelo tratamento estatístico dos resultados e sua interpretação, para além de toda a colaboração na respetiva publicação, apoio fundamental na execução deste trabalho.

À Dra. Mariana Couto e novamente à Dra. Patrícia Mota por terem sido as primeiras autoras de dois dos artigos incluídos nesta dissertação.

À minha familia, por todo apoio, carinho, paciência e compreensão pelas inúmeras horas de ausência dedicadas à elaboração deste trabalho, sem o que este não teria sido possível.